The Journal of Comparative Pathology and Therapeutics

Vol. 65. No. 1. Price Seventeen Shillings and Sixpence

1. The columns of the Journal are open for contributions recording original research in veterinary science.

2. The Editorial Board accepts papers bearing on the above subject on the understanding that they are not being offered elsewhere for publication. It reserves the right to reject any papers submitted to it for publication.

3. It is essential that all articles should be concise. Any necessary allusions to matter already published should be brief and limited strictly to those relevant to the satisfactory exposition of the author's own work.

4. Citation of literature in the text should be accompanied by the year of publication. Authors' names (followed by year, publication, etc.) should be arranged alphabetically in a list at the end of the paper.

5. Papers for publication, exchange journals, etc., should be sent to Prof. T. J. Bosworth, Royal Veterinary College, London, N.W.1, or to any member of the Editorial Board.

6. Correspondence concerning business matters should be addressed to the Publishers, The University Press of Liverpool, Liverpool, 3.

7. The net price of each volume of four numbers is £3 paid in advance to the publishers. Single numbers may be obtained at a price of 17s. 6d. each.

Additional copies and reprints ordered before the issue is printed may be obtained from the publishers.

CONTENTS

PAGE

The Use of Blood Culture in the Routine Diagnosis of Anthrax. By P. D. Thomson 1

The Marrow Cells of Normal Sheep. By C. S. Grunsell 8

Staphylococcal Pyaemia in a Wild Hare (*Lepus europaeus occidentalis*). By A. McDiarmid 17

Serum Calcium and Magnesium and Plasma Phosphate Levels in Normal Parturient Cows. By E. W. Moodie, A. Marr and A. Robertson 20

Observations on Experimental Fowl Typhoid. By H. Williams Smith 37

The Chemotherapy of Experimental Fowl Typhoid in Fowls (*Gallus domesticus*) By H. Williams Smith 55

The Blood Groups of the Pig. I. The Interaction of Pig Red Cells of Group A and the Naturally Occurring A Iso-Antibody in the Serum of Pigs of Blood Group O. By Ruth Saison, R. F. W. Goodwin and R. R. A. Coombs 71

The Blood Groups of the Pig. II. Red Cell Iso-Antibodies in the Sera of Pigs Injected with Crystal Violet Swine Fever Vaccine. By R. F. W. Goodwin, Ruth Saison and R. R. A. Coombs .. 79

THE USE OF BLOOD CULTURE IN THE ROUTINE DIAGNOSIS OF ANTHRAX

By

P. D. Thomson

Veterinary Laboratory, Ministry of Agriculture, Weybridge.

INTRODUCTION

In 1950, while working with R. M. Loosmore at Weybridge, the author was introduced to the technique of incubating *B. anthracis* in fresh defibrinated bovine blood as a method of demonstrating capsule formation by that organism. Later, it was decided to carry out a comparison of the results obtained by this technique with those of the biological test in guinea pigs. Cases from the field which could not be confirmed by microscopical examination of the smears submitted and which, therefore, required further investigation, were used as the basis of the comparison.

The technique was also tried out along with guinea pig and mouse inoculation in the process of screening feeding stuffs and other materials for anthrax.

METHODS

For those suspected cases of anthrax from the field which are microscopically negative and required further investigation, all that is necessary is to add approximately 2 ml. of fresh defibrinated bovine blood to the glass tube in which the swab is contained, replace the swab and incubate at 37°C. Positive results have been obtained with as little as $3\frac{1}{2}$ hours incubation, but the usual procedure has been to incubate the swab in the late afternoon and remove the following morning (about $17\frac{1}{2}$ hours), although checks at six hours have generally proved equally satisfactory. Before incubation, the swab is used to inoculate a guinea pig.

In the morning, the swabs are removed from the incubator, smears are made, dried in air, fixed by heat and stained with a 1 per cent aqueous solution of methylene blue. In the positive cases, capsule formation has been generally good and quite often extremely well-marked. This was established in earlier experiments with swabs from a small number of cases found positive on microscopical examination and has proved to be so throughout the series of comparisons. A small number of cases have shown similar results using defibrinated sheep blood and also sera from cattle, horse, sheep, donkey and pig. Defibrinated bloods and sera have been used up to ten days following collection, with satisfactory results. Furthermore, a small number of trials using anthracoids have failed to show capsule formation with any of the strains in any of the bloods or sera used.

When screening feeding stuffs for the presence of anthrax, the technique is used in conjunction with a modification of the method described by Jones (1942). The material for examination is first reduced to a suitable size—in the case of bones by grinding in a hand-mill—and a 15 g. sample transferred to a 300 ml. Erlenmeyer flask with 150 ml. of physiological saline solution. The mixture is shaken several times during a period of three to four hours and stored in a refrigerator overnight. On the following day the flask is again shaken several times and put in a water bath containing cold tap water, which is heated till the contents of the flask attain a temperature of 65°C. This temperature is maintained for a period of five minutes, the flask is allowed to cool to room temperature, the contents are filtered through two layers of butter muslin and the filtrate distributed into universal containers to be spun for ten minutes at about 3,000 r.p.m. in a horizontal centrifuge. The deposits are suspended in physiological saline, recentrifuged, and made up to a final volume of approximately eight ml. One ml. is inoculated into blood, one ml. into one guinea pig, three ml. into a second guinea pig and doses ranging from 0.5 to 0.2 ml. into six mice, the animal inoculations being done subcutaneously.

Diagnosis was based on the demonstration of typical anthrax organisms in smears from the blood culture, of the swab and from smears from the site of inoculation, heart blood and spleen of the animals. Cultures on blood agar plates were also made from the same organs .

So far, some 273 samples have been examined. Mice varying from three to twelve were used in only 202 examinations before it was decided that six was a suitable number to employ with a reasonable chance of recovering anthrax should it be present in the sample under examination.

RESULTS

Table 1 shows the results of the series of comparisons made between cultures in defibrinated blood and inoculation of the guinea pig with the swabs from the microscopically negative cases of suspected anthrax sent in for diagnosis. It will be observed that the

TABLE 1

Diagnosis	*Blood culture*	*G. pig inoculation*
Number confirmed	48	15
Number not confirmed ..	675	708
Totals :	723	723

blood culture technique yielded rather more than three times as many positive results as the guinea pig inoculation method. A point of considerable importance is that the biological method did not detect any that had not already been confirmed by blood culture.

At various times between August 1952 and October 1953, the period during which the comparisons were carried out, blood cultures, which had shown the presence of anthrax organisms, but for which the corresponding guinea pig had given a negative result, were used to inoculate other guinea pigs. The original blood culture from the swab was retained on the bench at room temperature until required. As some gave negative results in these guinea-pigs, further checks were made at shorter intervals on blood cultures giving positive smear. Table 2 shows the results of these re-inoculations, the age of the culture being that period, in days, which elapsed between adding blood to the original swab and the day on which the same swab was used for inoculation of a second guinea pig. Diagnosis in the guinea pigs was by examination of heart-blood smears, where applicable. The distribution of deaths in relation to the age of the cultures appears to be of interest.

TABLE 2

Age of culture in days	*Result of inoculation of guinea pig with blood culture from swab*
13	Negative. Guinea pig. Survived
12	,, ,, ,, ,,
8	,, ,, ,, ,,
5	,, ,, ,, ,,
5	,, ,, ,, ,,
5	Positive. Death 96-120 hours
3	,, ,, 36- 48 ,,
3	,, ,, 48- 72 ,,
2	,, ,, 72- 96 ,,
1	,, ,, 120-144 ,,
1	,, ,, 18- 24 ,,
1	,, ,, 24- 48 ,,
1	,, ,, 72- 96 ,,
*1	Negative. ,, 24- 36 ,,

*This animal was subjected to a post-mortem examination which showed that death had apparently been due to pneumonia.

Table 3 shows the results from blood culture, guinea pig and mice inoculations in the examinations of feeding stuffs for anthrax.

The figures do not include eight mice not examined as they had been rendered unsuitable for bacteriological examination because they had been wholly or partly devoured. In such mice, attempts were made to diagnose anthrax by cultural methods, but the plates were almost always overgrown with rapidly growing "spreaders." In these instances the presence of anthrax in the feeding stuff was

TABLE 3

	G. pig	*Mouse*	*Blood Culture*
No. positive	7	40	4
No. indefinite ..	0	3	3
No. negative ..	539	1159	266
	546	1202	273

confirmed in their companions when they died usually twenty-four to seventy-two hours afterwards. Some of the mice which died after inoculation with a feeding stuff suspension contaminated with anthrax failed to show anthrax as the cause of death, many having

TABLE 4

	Anthrax present	*Anthrax not present*
Cereals	0	56
Compound foods (cakes, meals, etc.) ..	3	109
Bone, bonemeal, hoof, horn	13	50
Dried blood, carcase, meat and fish-meals	0	16
Fertilisers, mineral mixtures, processed bone	0	15
Faeces from "Infected Premises"* ..	1	10
Totals :	17	256

*"Infected Premises"—where anthrax has been confirmed in an animal thereon.

TABLE 5

	Compound feedingstuffs	*Bones etc.*	*Faeces*	*Totals*
All three methods ..	0	2	0	2
G.p. only	1	1	0	2
Mouse only	1	6	0	7
Blood culture only ..	0	0	0	0
G.p. and mouse ..	1	2	0	3
G.p. and blood ..	0	0	0	0
Mouse and blood ..	0	2	1	3
	3	13	1	17

died of anaerobe infections: they were listed as "negative." There were five similar cases among the "negatives."

The types of feeding stuffs and other material examined, together with the results of examination are shown in Table 4.

Table 5 shows the breakdown of the above figures and the number of samples found positive by the inoculation of guinea pigs and mice, by blood-culture and by the various combinations of all three. No account has been taken of those cases where the blood culture was indefinite either alone or in combination with others.

DISCUSSION

The six failures to infect guinea pigs with cultures of original swabs in defibrinated blood, which had been retained for varying periods of time after proving positive on smear examination, are of some interest. It has been noticed with swabs containing anthrax but heavily contaminated with other organisms, which was frequently the case with swabs from the field, that the addition of defibrinated blood to the swab tube followed by incubation overnight would give a positive smear the following day, but also, that the anthrax bacilli appeared to die out in about five to seven days, even at room temperature, although "ghost" forms might persist for several days after typical organisms ceased to be in evidence in stained smears. After a period of about a week, according possibly to the degree or type of contamination, the blood culture appeared to have become non-infective for the guinea pig by subcutaneous inoculation.

It is stated by Topley and Wilson (1936) that putrid blood often proved non-infective in early attempts at experimental transmission. In the experiments reported here, it may well be, that the failure to infect the five guinea pigs which survived was due to the use of an inoculum containing only dead organisms or dying organisms much reduced in virulence, but another point which may be of some importance is that a minimum lethal dose level was not achieved. Gitter (1953) at Weybridge, by adding a specific number of spores to a feeding stuff and using a suspension therefrom for inoculation, has estimated that approximately twenty viable spores from a virulent culture were necessary to set up a lethal infection by the intraperitoneal route. Keppie, Smith and Harris Smith (1953) showed that 1×10^4 spores inoculated by the intraperitoneal route were necessary to kill guinea-pigs of 300 to 350 g. in weight. They further demonstrated that, whereas 50 spores in 20 ml. of gelatin-Locke solution were non-lethal for 30 guinea-pigs, the same number combined with 20 ml. of "aggressin" caused the death of 15/15. It is possible, therefore, that some factor, or factors, were present in the feeding stuff used by Gitter which had an aggressin-like effect. Jonescu-Mihaiesti (1938) has also shown that there is quite a wide variation in the M.L.D. for mice, so that one might reasonably

assume some degree of variation of susceptibility to infection among guinea pigs.

Many of the "further investigations" concerned in this series of comparisons were in pigs which died during the period when the enteric form of anthrax was fairly common and, such cases usually provided the highest degree of contamination encountered. In these cases anthrax bacilli were often scanty in the smears sent in and it is certain that many of the accompanying swabs had been taken from the same source as the smears and were, on that account, somewhat deficient in numbers of organisms or spores. That this was so was proved by the examination of smears made from positive cultures in blood of these swabs, where a more diligent search was necessary than is customary for smears from cattle. In fact, in two cases proved positive by blood culture, the original smears from the field were re-examined very carefully and widely scattered anthrax bacilli were eventually found, but they were so few that it required about twenty minutes search per smear to find them. Three or four other smears were re-checked in this way without any anthrax bacilli being seen.

One other point of interest is that on a few occasions anthrax has been demonstrated in smears and cultures from the site of inoculation only. In these cases, a heavy local anaerobe infection had apparently caused the death of the animal before the anthrax bacilli had reached a sufficiently advanced stage of multiplication to permit invasion of the blood stream. It is possible that under such circumstances where the bacilli are scanty and there are contaminating organisms present, the growth of the latter provides unfavourable conditions for multiplication of the anthrax organisms. Such conditions arise in culture in the blood medium when the culture is retained for a few days and the anthrax bacilli die out as a result.

CONCLUSIONS

A blood culture technique is described as an aid to diagnosis in indefinite cases of suspected anthrax in the field. The use of defibrinated blood or serum in such cases has the advantage that it is cheap, simple and apparently more sensitive than guinea pig inoculation. It does not suffer from one important disadvantage of the biological test namely the occasional death of the guinea pig before the statutory period of six days through the presence of other pathogens.

In the examination of feeding stuffs and other materials, however, it would appear that the inoculation of six mice is superior to either two guinea pigs or one blood culture, but quite clearly, more positive results are obtained by combining all three methods.

ACKNOWLEDGMENTS

My thanks are due to the Director, Dr. A. W. Stableforth, for permission to publish this paper and to Mr. J. R. Hudson for his continuing

interest, helpful suggestions and assistance at various times during the course of this work. I am also indebted to my technical assistant, Ian Hopkins, for willing help at all times.

REFERENCES

Gitter, M. (1953). *Private communication.*
Keppie, J., Smith, H., and Harris-Smith, P. W. (1953). *Brit. J. exp. Path.*, **34**, 486.
Jones, E. R. (1942). *J. Path. Bact.*, **54**, 307.
Jonescu-Mihaiesti, C. (1938). *Quart, Bull. Hlth. Org. L.o.N.*, **7**, 827.
Topley and Wilson, (1936). *Principles of Bacteriology and Immunity.*, p. 1374 2nd Edition, Edward Arnold & Co, London.

[*Received for publication, April 2nd,* 1954]

THE MARROW CELLS OF NORMAL SHEEP

By

C. S. Grunsell

Department of Hygiene and Preventive Medicine

Royal (Dick) School of Veterinary Studies, University of Edinburgh.

INTRODUCTION

In a previous paper (Grunsell, 1951) a technique for marrow biopsy in sheep was described and haemomyelograms and myeloid erythroid ratios for ten sheep determined; however, no reference was made to cell morphology. The object of this paper is to describe the morphology of the marrow cells of the sheep. Two methods of assessing marrow activity for use in conjunction with the haemomyelogram and myeloid erythroid ratio are also described. The first is based on the grading of cellularity of marrow spreads; the second, on the examination of histological sections of marrow flecks embedded according to a procedure described by Campbell (1948). The morphology of eosinophils in sheep bone marrow has been studied by Ringoen (1938), but, apart from an account by Goodall (1907) of the structure of certain cells, there has been no comprehensive description of the histology of the sheep's marrow.

MATERIAL AND METHODS

Description of the cells. The description of cell morphology is based on the examination of marrow spreads from six sheep prior to slaughter. These animals were found at post-mortem examination to have no gross pathological lesions. The techniques used in securing biopsy samples of the marrow and the preparation of the spreads have already been described (Grunsell, 1951). The cells were classified according to a scheme of nomenclature proposed by Israels (1941). For the description of the haemocytoblast twenty cells were examined; in the case of myeloblast, promyelocyte, proerythroblast, plasma cell and reticulum cell at least 100 cells were studied; for each of the other cells of the marrow the description was based on the examination of at least 1,000 cells. Haemocytoblast, proerythroblasts and all cells younger than metamyelocytes were measured by a micrometer eyepiece. For the remaining cells the images were projected on to a sheet of paper and measured by a ruled scale. Whilst studying the morphology of the cells a record was kept of those seen in mitosis. The stages differentiated were prophase, metaphase, anaphase and telophase (Maximow and Bloom, 1938).

Grading of the cellularity of marrow spreads. It was observed that the technique which has been described for spread preparations (Grunsell, 1951) produced a characteristic disposition of the marrow cells, the majority of which were seen surrounding the central reticular syncytium. The other two positions in the spread in which cells appeared in any numbers, were within the walls of the syncytium and along the edges of the spread. The grading of the cellularity of the spreads was based on the density of the cells in these three positions. The standards were drawn up from the examination of marrow spreads from over 100 sheep. This

number included normal sheep and sheep having either hyper- or hypoplastic marrows. To assess the cellularity of a sample of marrow at least two spread preparations were surveyed. Examination was carried out using the 2/3 objective with x 10 eyepieces.

The cellularity of the marrow spreads was classified into six grades. The criteria used in this classification are shown in the table.

TABLE 1

THE CLASSIFICATION OF THE CELLULARITY OF MARROW SPREADS

Grade	*Density of cells*		
	Within the walls of the syncytium	*Around the syncytium*	*Along the edges of the spread*
O	—	— or +	— or +
I	—	+	++
II	—	++	++
III	—	+++	+++
IV	++	++++	+++
V	+++	++++	+++

KEY

1. Absent	—
2. Cells present but widely separated	+
3. A density of cells comparable with that of leucocytes along the edges of a normal blood film	++
4. Denser masses of cells but not touching each other	+++
5. Dense masses of cells touching each other	++++

Preparation of histological sections from marrow flecks. The method employed was described by Campbell (1948) for the histological preparation of bone marrow particles. The sections of normal and hypoplastic marrow shown as Figs. 9 and 10 respectively were prepared using this technique.

RESULTS

Some of the features of the cell morphology described below are illustrated in Figs. 1 to 8.

Primitive Cell-types

Haemocytoblasts. These cells are rare. The irregularly shaped nucleus occupied the greater part of the cell and consisted of an open pink network with as many as five nucleoli. The irregular cell outline enclosed a pale grey blue cytoplasm with no inclusions.

Haematogones. In all spreads examined a number of cells about the size of intermediate normoblasts were seen. Their red nucleus was surrounded by a narrow ring of pale blue cytoplasm. These cells were classified as haematogones. (Fig. 4n.)

Granuloblasts (Figs. 1 to 5). The term granuloblast is used in this paper to include all precursors of the granular leucocytes. Primitive undifferentiated myeloblasts and promyelocytes were identified and from the staining affinity of the granules and nuclear structure it was possible to classify the remaining granuloblasts into neutrophilic, eosinophilic and basophilic, myelocytes and metamyelocytes. The neutrophilic metamyelocytes were sub-divided into juvenile and stab forms according to Schilling (1929).

The nucleus in the myeloblast (Fig. 2a) and in the promyelocyte was central in position and round in shape, having a finely reticular structure and staining pink. It contained up to five nucleoli. The cytoplasm was pale blue in both the myeloblast and the promyelocyte, and in the latter cell contained small azure granules. In about half the myeloblasts examined a clear perinuclear halo was visible. (Fig. 1a.)

It was found that the morphology and staining affinity of the nucleus and cytoplasm in the myelocytes and metamyelocytes did not differ in the three series of granuloblasts. The nuclear structure described for the myeloblast and promyelocyte was repeated in myelocytes and juvenile metamyelocytes, but the nuclei were eccentric. (Figs. 1b, 2c and 3f.) Some myelocytes contained up to three nucleoli. These were never seen in metamyelocytes. The round outline of the nucleus of the myelocyte became indented at the juvenile metamyelocyte stage, the degree of indentation varying from a slight depression to a deep indentation giving the nucleus the shape of a wide U. (Fig. 2c). In the stab metamyelocyte the nucleus was S, U or W shaped, and the structure consisted of knots of basophilic chromatin in a pink staining background.

The pale blue cytoplasm in both myelocytes and juvenile metamyelocytes was less basophilic than in the myeloblasts and promyelocytes. In the metamyelocytes where the nuclear outline was only slightly depressed a cytocentrum was often visible at the 'hilus' of the nucleus. This was most frequently seen in the neutrophilic metamyelocytes. The cytoplasm of the stab metamyelocyte was yellow to faint pink in colour.

The disposition of the granules in the cell was related to the age of the cell as estimated by its nuclear outline. In the myelocytes the granules were distributed throughout the cytoplasm and extended over the nucleus obscuring the nuclear outline. (Fig. 3f). This was particularly noticeable in the basophilic myelocytes giving the cell a pyknotic appearance and making identification difficult. (Fig. 4g). As the nuclear indentation became deeper, the number of granules lying over the nucleus was reduced so that in metamyelocytes with frank nuclear indentation the granules were confined to the cytoplasm. In the neutrophilic series two types of granule were demonstrated—a small azure granule and a colourless neutrophilic granule. The azure granule did not persist beyond the early form of

juvenile metamyelocyte. In the stab metamyelocyte the neutrophilic granules were much smaller and stained reddish brown.

The eosinophilic myelocyte (Fig. 3f) contained four types of granule. They were classified on their staining affinity as eosinophilic, azure, polychromatophilic and basophilic. The eosinophilic granules were most numerous; next in prevalence were the azure granules of two distinct sizes; the polychromatophilic and basophilic granules were rare. In the metamyelocytes the small azure, the basophilic and the polychromatophilic granules had disappeared. The basophilic granuloblast contained only basophil granules but a large and a small type were recognised in both myelocytes and metamyelocytes.

The mature lobulated forms of all three series were seen in the marrow and were indistinguishable from the cells in the peripheral blood described by Fraser (1929-30), Holman (1944) and others. (Fig. 5e).

Erythroblasts (Figs. 1, 5 and 7).

Throughout the series the nucleus occupied most of the cell and was round in shape. The nuclear structure of the proerythroblast (Fig. 1h) consisted of a coarse dark purple network of basophilic chromatin against a pink background and contained three to five nuclei. The pattern of the nucleus of the early normoblast (Fig. 7i) was made up of fine strands of chromatin arranged in circular whorls. The staining affinity was less basophilic than the proerythroblast and gave the nucleus a dark pink colour. No nucleoli were visible. In the nucleus of the intermediate normoblast the chromatin aggregated in darkly basophilic clumps which stood out like the spokes of a wheel against the pink background. (Fig. 5j). This structure was lost in the late normoblast (Fig. 6k) in which the nucleus was a deep pyknotic mass staining purple or black. The nuclei of the proerythroblast and the early normoblast were of similar size but the progressive aggregation of chromatin seen in the intermediate and late normoblasts was accompanied by a reduction in the size of the nucleus.

The cytoplasm of the proerythroblast formed a dark narrow ring round the nucleus. In the other members of the series the overall tendency was for the staining affinity of the cytoplasm to change from basophilic to oxyphilic with the maturation of the nucleus. However, within each of the three groups early, intermediate and late, wide variation in the colour of the cytoplasm was found to be possible. The table below shows the approximate incidence of the different staining affinities in the cytoplasm of the early, intermediate and late normoblasts.

Immature Erythrocytes

Two forms of immature erythrocyte were noted in the spreads. These were either fully haemoglobinated cells showing punctate basophilia, or cells which were polychromatophilic, the former being

TABLE 2

THE STAINING AFFINITY OF THE NORMOBLASTS AT DIFFERENT STAGES OF MATURATION

Cell	*Staining affinity of cytoplasm. Approximate percentage of cells showing:*			
	Deeply basophilic	*Basophilic*	*Polychromatophilic*	*Oxyphilic (Fully haemoglobinated*
Early Normoblast	50	25	25	—
Intermediate Normoblast	—	25	75	—
Late Normoblast	—	—	90	10

of much more frequent occurrence than the latter. Most of the cells showing polychromasia were macrocytes, whilst those with punctate basophilia were of the same size as the mature erythrocytes. Nuclear remnants occurring in the erythrocytes as Howell-Jolly bodies, or Cabot rings, were not observed at any time.

Plasma Cells

Both mature and immature plasma cells were seen. The nucleus was round, eccentric and occupied one-third to one-half of the cell in all stages of maturity. The nucleus of the immature cell consisted of a loose pink reticulum in which nucleoli were visible and was surrounded by a slatey blue cytoplasm. In the mature cells the nuclear chromatin was deeply basophilic and clumped into a cart-wheel formation. The cytoplasm was royal blue and contained numerous vacuoles and almost all mature plasma cells included an unstained area in the cytoplasm against the medial pole of the nucleus. (Fig. 6l).

Reticulum Cells

The nucleus was round or oval and composed of a fine reticulum of pink chromatin. Nucleoli were often visible. The cytoplasm was smokey grey in colour, frequently containing fat droplets and green staining pigment. Dark pyknotic structures, indistinguishable from the nuclei of late normoblasts, were often seen as cytoplasmic inclusions. (Fig. 7m).

Megakaryocytes (Fig. 8o). The chromatin of the nucleus of these cells was deeply basophilic, and arranged in streaks and clumps. Both lobulated and non-lobulated nuclei were seen. All degrees of lobulation were represented and in the most advanced stage the lobes were separated from each other. The extent of the cytoplasm increased with the lobulation of the nucleus and where the nucleus was divided into two distinct lobes the cytoplasm was very extensive. The presence of purple staining particles distributed throughout the

pink background of the cytoplasm gave it a speckled appearance. In cells where the nucleus was non-lobulated and where lobulation was just starting a ring of homogeneous dark blue cytoplasm separated the nucleus from the main mass of speckled cytoplasm. All types of marrow cell were observed adhering to the cytoplasm of the megakaryocytes.

Lymphocytes and monocytes. No immature lymphocytes or monocytes were found in the marrow spreads examined and even the mature forms of both series were of infrequent occurrence (Grunsell, 1951). Those seen had the morphology of the lymphocytes and monocytes of the peripheral blood as described by Fraser (1930), Holman (1944) and others.

Cell Measurements

The means and ranges for ten sheep were detailed in Table 2 of a previous article (Grunsell, 1951). Particular attention is directed to column 4 of this table which gives the means and the ranges for the cells described in the present article. The measurements of the mature leucocytes are not included as they did not differ materially from the measurements which have been published for these cells in the peripheral blood.

TABLE 3

THE RELATIVE FREQUENCE OF THE FOUR PHASES OF MITOSIS

	Prophase	*Metaphase*	*Anaphase*	*Telophase*
Granuloblasts	31·6%	21·0%	15·8%	51·6%
Erythroblasts	41·4%	35·6%	11·5%	11·5%

Mitosis

The percentage of cells seen in mitosis varied from 0·2 per cent to 1·6 per cent with a mean value of 0·84 per cent. Division was observed more commonly in erythroblasts than granuloblasts, viz. 89 per cent of the cells seen in mitosis were erythroblasts. Division was observed in all ages of erythroblast but not beyond the stage of myelocyte in the granuloblasts. Of the erythroblasts seen in mitosis 1·9 per cent were proerythroblasts, 21·0 per cent early normoblasts, 72·6 per cent intermediate normoblasts, and 4·5 per cent late normoblasts. Of the granuloblasts 26·3 per cent were myeloblasts, 68·4 per cent neutrophilic myelocytes and 5·3 per cent eosinophilic myelocytes. Table 3 shows the relative frequence with which the stage of mitosis were seen in the two groups of cells.

DISCUSSION

Israel's (1941) scheme of nomenclature was applicable to the marrow of the sheep. It is generally acknowledged that it is often

difficult to differentiate the haemocytoblast, the myeloblast and the proerythroblast. This difficulty arose with some of the primitive cells seen in the spreads but it was found that each of the three cells in question had characteristics which distinguished it. The haemocytoblast was differentiated by the finer and less basophilic reticulum of the nucleus and from the fact that the irregular cell outline enclosed a more extensive cytoplasm than either the myeloblast or the proerythroblast. The myeloblast resembled more nearly the haemocytoblast than the proerythroblast but it was distinguished by having less cytoplasm and a coarser and more basophilic nuclear pattern. In the nucleus of the proerythroblast the nuclear chromatin appeared in thicker bands and was more deeply basophilic, while the cytoplasm was less extensive and royal blue in contrast to the light blue cytoplasm of the haemocytoblast and the myeloblast. Columns of erythroblasts were commonly seen in the spreads and when cells with the above characteristics appeared along with the more mature members of the series, their identification as proerythroblasts received additional confirmation.

The granules of the neutrophilic granuloblasts showed affinity for neither acid nor basic components of the stain and were classified as truly neutrophilic (Ehrlich, 1879). This agreed with the findings of Holzel (1939) and Marcato (1941) for cattle and is of some interest in view of Cowdry's statement (1938) that in most animals there is a tendency for these granules to stain with acid dyes when they are termed pseudo-eosinophilic. The basophilic and polychromatophilic granules found in the eosinophilic myelocytes in this study of the sheep's marrow were not described by Holzel or Marcato as occurring in cattle, but Ringoen (1938) recorded them in the eosinophils of both the bone marrow and the haemolymph nodes of sheep. Ringoen postulates that they represent stages in the ripening of the eosinophilic granule and the fact that these granules were seen in my examinations in myelocytes but never in metamyelocytes may support his theory.

In the erythroblasts, although the younger cells normally had a more basophilic cytoplasm, the staining affinity of the cytoplasm did not bear a very close relationship to the stage of maturation of the nucleus. Thus, for example, cells which by their nuclear structure were classified as early, intermediate and late normoblasts were all on numerous occasions observed with a polychromatophilic cytoplasm. This finding supports the opinion of Dacie and White (1949), Israels (1941) and others, that the progressive pyknosis and diminution in size of the nucleus of the erythroblast is a more reliable guide to cell maturity than is the state of ripening of the cytoplasm. Complete haemoglobination of the cytoplasm was only seen in late normoblasts but even at this stage of maturity the cytoplasm was much more commonly polychromatophilic. Thus, in attempts to use cytoplasmic tint as an indication of the haemoglobin content of

erythroblasts, as, for example, in iron deficiency, this fact would have to be taken into account. Erythrocytes showing punctuate basophilia were much more numerous than were those showing polychromasia, but no significance can be attached to the finding as Farmer and Maizels (1939) have shown that the relative incidence of these two forms depends on the duration of staining and fixation. The presence in the cytoplasm of the phagocytic reticulum cells, of what appeared to be the nuclei of late normoblasts, suggests that exclusion may be one method by which the nucleus of the erythroblast is lost in sheep. An alternative explanation for the presence of these inclusions is that they represent the remnants of degenerate late normoblasts which have never reached maturity.

A technique for quantitative cell counts on aspirated marrow has been described by Isaacs (1937) but Reich and Kolb (1942) have shown the results to be so inaccurate as to be of little value. A scheme for the assessment of marrow cellularity similar to that described here has been produced by Propp and Schwind (1944). Their method, however, was designed for use in marrow blood films and was therefore not applicable to spreads prepared by crushing marrow flecks. No previous attempt has been made to measure the cellularity of marrow spreads and it is considered that the scheme presented for grading the cellularity in these preparations, augmented where necessary by the examination of fleck sections, should considerably increase the information to be obtained from haemomyelograms and maturation curves.

CONCLUSIONS

The morphology of the marrow cells of the sheep has been described.

Two methods for measuring marrow activity in samples obtained by sternal biopsy are presented.

ACKNOWLEDGMENTS

I wish to express my thanks to Professor G. F. Boddie, in whose department this work was carried out, for his advice and encouragement, and Mr. R. C. W. S. Hood for the preparation of the illustrations to this paper.

REFERENCES

Campbell, A. C. P. (1948). *J. Path. Bact.*, **60**, 633.
Cowdry, E. V. (1938). *A Textbook of Histology.* Kimpton; London.
Dacie, J. V., and White, J. C. (1949). *J. clin. Path.*, **2**, 1.
Ehrlich, P. (1879). Quoted, Bunting, C. H. (1938). "*The Polymorphonuclear neutrophile Leucocyte.*" Chapter in Hal Downey's Handbook of Haematology. Vol. 1. Hoeber; New York.
Farmer, S. N., and Maizels, M. (1939). *Biochem. J.*, **33**, 280.
Fraser, A. C. (1929-30). *Rep. Dir. Inst. anim. Path., Camb.*, **1**, 114.

Goodall, A. (1907). *J. Path. Bact.*, **12**, 191.
Grunsell, C. S. (1951). *Brit. vet. J.*, **107**, 16.
Holman, H. H. (1944). *J. comp. Path.*, **54**, 26.
Holzel, E. (1939). *Inaug. Diss.*, Universitat, Berlin.
Isaacs, R. (1937). *Amer. J. med. Sci.*, **193**, 181.
Israels, M. C. C. (1941). *Lancet*, **2**, 207.
Marcato, A. (1941). *Nuova Vet.*, **19**, 173.
Maximow, A., and Bloom, F. (1938). "*Textbook of Histology.*" 3rd ed. Saunders; London.
Propp, S., and Sshwind, J. L. (1944). *Ann. intern. Med.*, **21**, 580.
Reich, C., and Kolb, E. (1942). *Amer. J. med. Sci.*, **204**, 496.
Ringoen, A. R. (1938). "*Eosinophile Leucocytes and Eosinophilia.*" Chapter in Hal Downey's Handbook of Haematology. Vol. 1. Hoeber; New York.
Schilling, V., Trans. by Gradwohl, R. B. H. (1929). "*The Blood Picture*," Kimpton; London.

[*Received for publication, May 13th,* 1954.]

LEGENDS TO FIGURES

Myeloblast	a
Neutrophilic Myelocyte	b
Neutrophilic Juvenile Metamyelocyte	c
Neutrophilic Stab Form Metamyeocyte	d
Neutrophilic Polymorphonuclear Leucocyte	e
Eosinophilic Myelocyte	f
Basophilic Myelocyte	g
Proerythroblast	h
Early Normoblast	i
Intermediate Normoblast	j
Late Normoblast	k
Plasma Cell	l
Reticulum Cell	m
Haematogone	n
Megakaryocyte	o
Cell in Mitosis	p

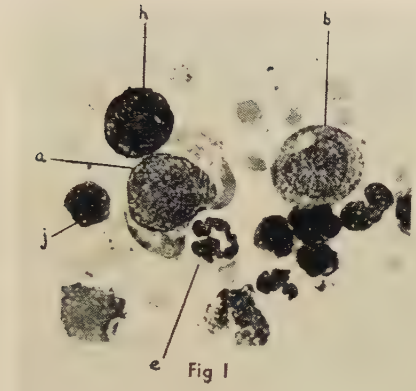


Fig 1

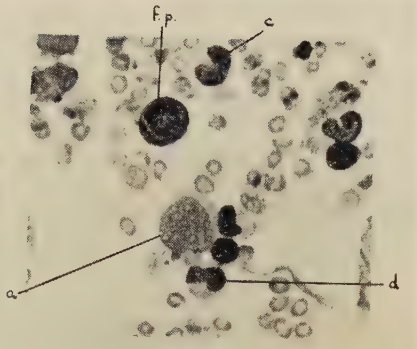


Fig 2

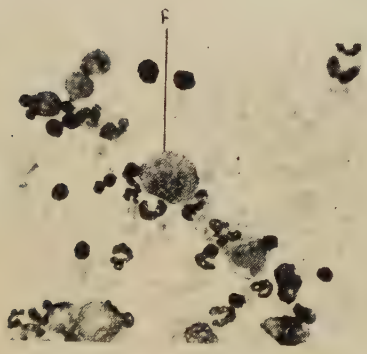


Fig 3

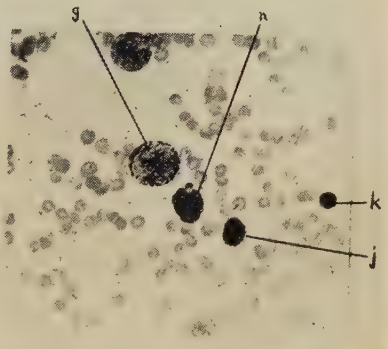


Fig 4

Fig. 1 × 600

Fig. 2 × 500

Fig. 3 × 350

Fig. 4 × 400

To face page 16

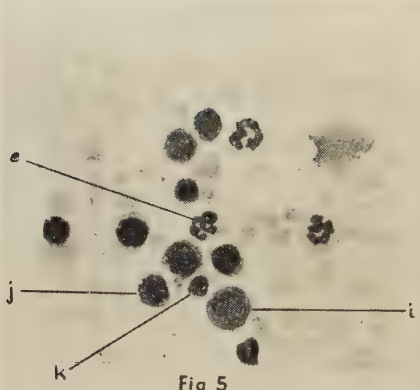


Fig 5

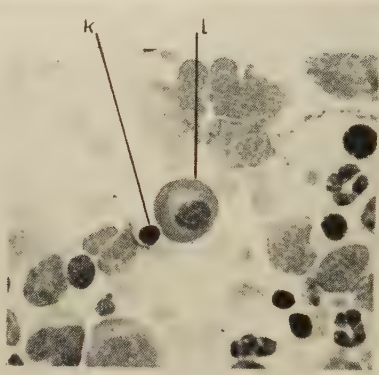


Fig 6

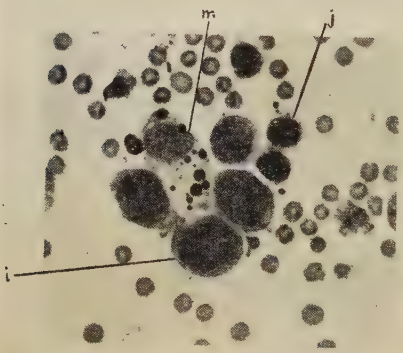


Fig 7

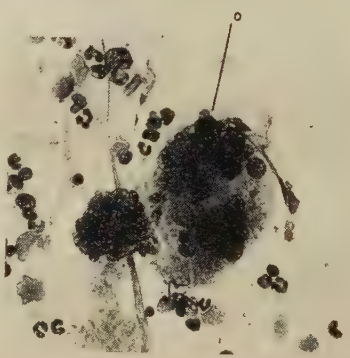


Fig 8

Fig. 5 × 500
Fig. 7 × 500

Fig. 6 × 500
Fig. 8 × 250

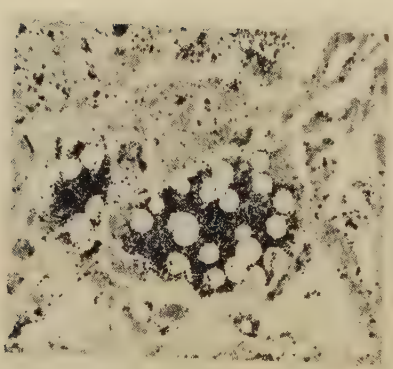


Fig 9

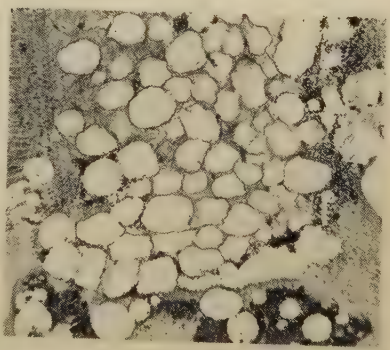


Fig 10

Fig. 9 × 75

Fig. 10 × 75

STAPHYLOCOCCAL PYAEMIA IN A WILD HARE (*LEPUS EUROPAEUS OCCIDENTALIS*)

By

A. McDiarmid

Agricultural Research Council, Field Station, Compton, Berks.

INTRODUCTION

Apart from suppuration following a variety of mechanical injuries such as previously inflicted gunshot wounds, abscesses have rarely been encountered in free-living wild hares in Britain. The purpose of this paper is to record the occurrence in the wild brown hare of an idiopathic pyaemic condition resulting from infection with *Staphylococcus aureus* and apparently unrelated to any external lesion.

DESCRIPTION OF CASE

Clinical History and Autopsy Findings

The hare was found alive, although unable to move, at the edge of a field of lucerne on the Berkshire Downs early in August, 1950. The symptoms indicated some form of paralysis of the hind limbs rather than a toxaemic condition. Several dead hares were found on this estate, but they were unfit for bacteriological examination. Numerous deaths had also been noticed in young hares on neighbouring farms during the summer months but unfortunately none was brought for post-mortem examination.

The principal macroscopic lesions involved the heart (Fig. 1) and kidneys. Small discrete abscesses varying in size were scattered throughout these tissues and the lesions were surrounded by a zone of inflammatory reaction. Other organs, especially the liver, showed slight congestion and petechial haemorrhages. In view of the symptoms of apparent paralysis particular examination was made of the immediate vicinity of the brain and spinal cord for the presence of a purulent lesion impinging on the meninges from the surrounding tissues but no such abnormality could be found. The main limb joints were unaffected. The skin was searched without result for any sign of external damage. No ticks were present.

RESULTS

A pure culture of a Gram positive coccus indistinguishable morphologically and culturally from *Staphylococcus aureus* was recovered from the myocardial and renal abscesses. Subsequent examination showed this organism to ferment, without gas, the following carbohydrates: galactose, glucose, lactose, maltose, mannitol, mannose, saccharose and trehalose. The strain was haemolytic ($\alpha\beta$ type) and coagulase positive; orange pigment was produced on Loeffler's medium and the organism was finally

identified as belonging to the 42D group by bacteriophage typing. A pyaemia could be produced experimentally in mice inoculated intravenously with suspensions of this organism.

Sections were prepared from the heart and kidney and stained by haematoxylin and eosin and also by Gram's method. Histologically the lesions were typical abscesses in which cocci were numerous. (Fig. 2).

DISCUSSION

Comparatively little is yet known in this country regarding the incidence of disease in free-living wild hares, but pyaemic conditions due to a variety of causal organisms are known to occur in the main species of hare (*L. europaeus*) on the European continent. Tularaemia is probably the most important disease because of the risk of transmission to the human population and it appears remarkable that, so far, it has not been found in our subspecies in Britain. A pyaemia of hares apparently due to a staphylococcus has also been described in Italy by Spinelli and Penso (1932). The writer's interest in the present case is two-fold.

Firstly, it is recognised that hares, in common with many species of wild life, undergo a definite fluctuation in population. Thus "hare years" occur followed by a sudden decline in numbers of this species; eventually a build-up takes place resulting once again in a peak point of density of population. In view of this situation it seems logical to assume that a sudden decrease in the numbers of hares may in many cases be associated with disease. The animal ecologists are, of course, well acquainted with this state of affairs and in particular Elton and his co-workers at Oxford have contributed considerably to our knowledge of marked fluctuations in a variety of animal species especially the vole, the partridge and the snowshoe rabbit. The diseases responsible for, or contributing to, these variations in a number of species have, however, rarely been investigated from the pathological or bacteriological aspects. The author has encountered a variety of infectious diseases in hares quite apart from the one described in this paper, and these infections may be far more important in controlling the numbers of this species than the hitherto generally accepted "adverse weather conditions," "availability of food" and other ecological factors such as predator-prey relationship.

Secondly, a specific staphylococcal infection occurs in lambs on t ck-infested farms in Britain (McDiarmid, 1946), and it has always seemed possible that a similar infection might be found in some species of free-living wild animal in this country. This case of pyaemia in a brown hare, although the animal was quite free from tick-infestation, simulates closely the classical disease in the lamb. Its occurrence in this specimen of wildlife again indicates the possibility of a widespread distribution of pathogenic organisms in nature.

Fig. 2

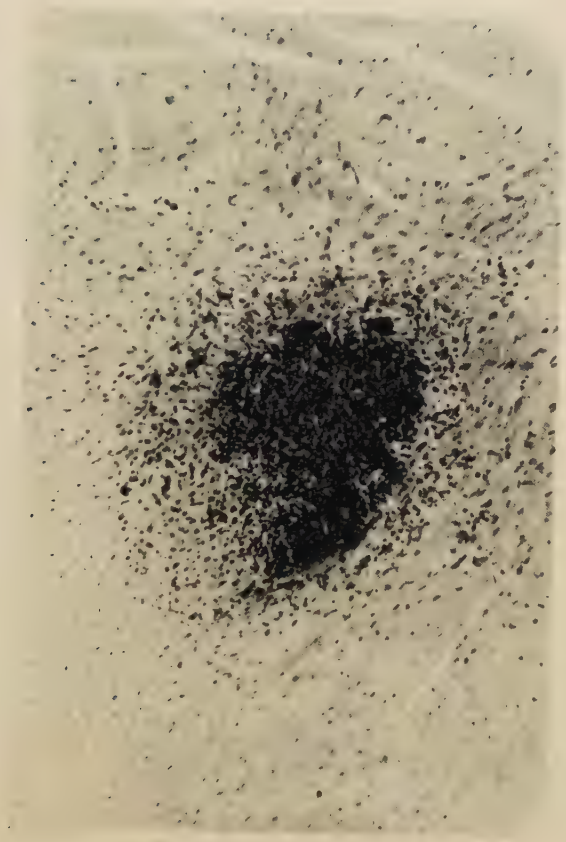


FIG. 2. Section of myocardium showing abscess formation. Stain H. and E.

Fig. 1

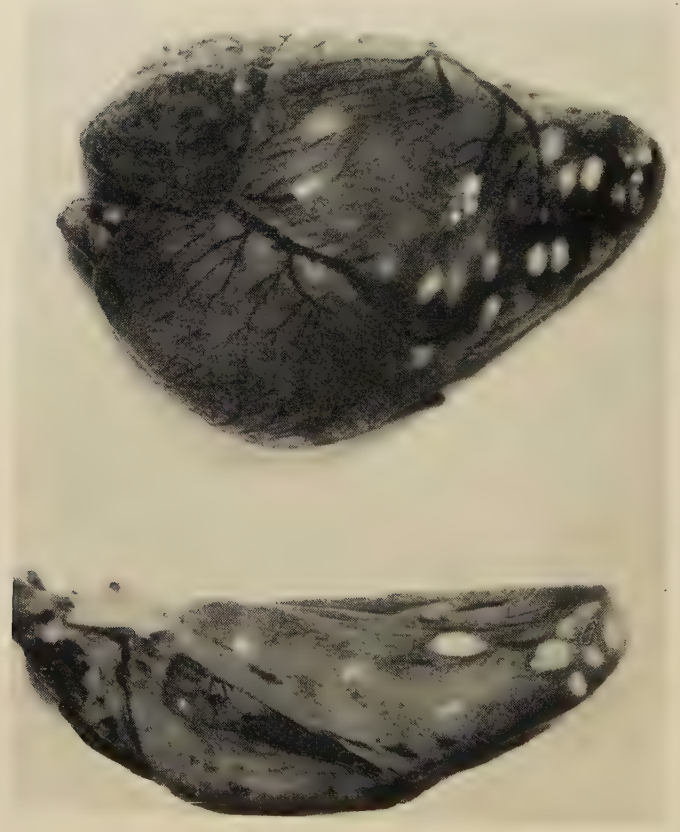


FIG. 1. Macroscopic lesions in the heart

To face page 19

When one considers that this type of staphylococcus (42D) is probably one of the commonest found associated with staphylococcal mastitis in the dairy cow and that in addition it is also apparently pathogenic for man (Smith, 1948), the connection between disease in wildlife and domestic stock becomes of even greater significance. In meat inspection, for example, the hare is rarely condemned except on account of putrefaction. (Thornton, 1949).

CONCLUSIONS

A pyaemia due to *Staphylococcus aureus* is described in the wild brown hare apparently unrelated to any external injury and in the absence of tick infestation.

The brown hare can act as a reservoir host for a species of organism potentially pathogenic to domestic stock and man.

ACKNOWLEDGMENT

Thanks are due to Mr. F. Summerfield, experimental officer at this Field Station, for preparing the photographs and to Dr. R. E. O. Williams, Central Public Health Laboratory, for determining the bacteriophage type.

REFERENCES

McDiarmid, A. (1946). *Vet. Rec.*, **58**, 103.
Smith, H. W. (1948). *J. comp. Path.*, **58**, 179.
Spinelli, A., and Penso, G. (1932). *Clin. vet. Milan*, **55**, 173.
Thornton, H. (1949). *Textbook of Meat Inspection.* Balliere, Tindall & Cox; London.

[*Received for publication, May 17th*, 1954.]

SERUM CALCIUM AND MAGNESIUM AND PLASMA PHOSPHATE LEVELS IN NORMAL PARTURIENT COWS

By

E. W. Moodie*, A. Marr,** and A. Robertson

Royal (Dick) School of Veterinary Studies, University of Edinburgh

INTRODUCTION

It is well known that the serum calcium is depressed in cases of bovine parturient paresis (Little and Wright, 1925; Dryerre and Greig, 1928). Fish (1929, 1930) focussed attention on the blood inorganic phosphate and Seekles, Sjollema and Van der Kaay (1932) on the serum magnesium. As a result there has been an extensive study of the variations in the blood calcium, magnesium and inorganic phosphate in normal parturient cows.

Various workers (Palmer, Cunningham and Eckles, 1930; Godden and Allcroft, 1932; Allcroft and Godden, 1934; Duckworth and Godden, 1940) have demonstrated a fall in the levels of serum calcium and plasma inorganic phosphate at parturition. Wilson and Hart (1932) noted a tendency for the parturient fall in blood calcium to be greater in older cows than in heifers. There is less general agreement of the behaviour of serum magnesium in the parturient cow. Seekles, Sjollema and Van der Kaay (1932) and Allcroft and Godden (1934) have reported a hypermagnesaemia in normal calving cows, while Hayden (1938) and Blood and White (1949) failed to observe any alteration in serum magnesium at parturition.

In contrast to the inorganic phosphate, the blood organic phosphate fractions have been the subject of only limited study. Little and Mattick (1933 a and b) are the only workers to have observed changes in these fractions at normal parturition, and Hayden (1938) failed to confirm them. Several workers (Fish, 1929, 1930; Barker, 1948; Robertson, Burgess, Marr and Milne, 1948; Robertson, 1949) have suggested that the blood phosphate levels may be of significance in clinical parturient paresis particularly in relation to response to treatment. The present investigation was undertaken because it was thought that a detailed study of the changes in the blood phosphate fractions in normal parturient cows, and the correlation of such changes with those in the serum calcium and magnesium, was desirable. Since parturient paresis rarely occurs before the third parturition it was decided to compare the magnitude of the changes in the blood fractions in parturient cows of different ages in order to test the hypothesis of Allcroft (1947) that the blood changes of parturient paresis are merely a pathological exaggeration of those occurring at normal parturition.

* Now at the Veterinary School, University of Queensland, Brisbane.

** Now at the University of Glasgow Veterinary Hospital.

MATERIALS AND METHODS

Animals. Thirty-two normal parturient cows were used. These were on two farms under the same management and were milked three times daily. The feeding practice was the same on both farms. The cows were mainly Ayrshires, although a few Shorthorn cross, Red Poll cross and Jersey cross cows were included. Of the 32, seven were having their first calf, eight their second, nine their third, and eight their fourth or subsequent calf.

Sampling. Blood samples were drawn from either the jugular or mammary veins. Approximately 90 ml. was collected at each sampling, 30 ml. being allowed to clot for calcium and magnesium estimations and 60 ml. oxalated for estimation of phosphate fractions.

The samples were taken from 14 to 7 days pre-partum until 10 to 20 days post-partum. As far as possible daily samples were taken from seven days before until seven days after calving, but as the investigation proceeded it was decided that the number of samples taken after calving must be increased if an adequate picture of the post-partum changes was to be obtained. In the later stages of the investigation, therefore, samples were taken every four hours on the first day, every eight hours on the second and twice on the third day following parturition.

Serum calcium was estimated by the Clark and Collip (1925) modification of the Kramer-Tisdall method. After precipitating the calcium, the supernatant fluid was used for estimating serum magnesium by the method of Denis (1922) modified according to Hawk (1947). The plasma inorganic phosphate was determined as soon after collection as possible, using the method of Fiske and Subbarow (1925). The organic phosphate estimations were adapted from the method described by Greenhill and Pollard (1935); the preparation of the extracts and the "blueing-up" were by the method of Fiske and Subbarow (1925). Colorimetric estimation of phosphates and magnesium were carried out in a "Spekker" absorptiometer.

All estimations were carried out in duplicate, and if the results differed by more than 5 per cent. the estimations were repeated.

Statistical analysis. t tests and analyses of variance were carried out as described by Goulden (1936).

RESULTS

General Trends

Figure 1 shows the mean serum calcium and magnesium and plasma inorganic phosphate fractions of all samples obtained from 32 cows, and the total acid soluble, lipid and total phosphates of all samples obtained from 18 of these cows, from seven days pre-partum until 10 days post-partum. As samples from different cows enter into successive means, the figure can only be regarded as demonstrating

the general trends in the behaviour of the different fractions at parturition.

Fig. 1

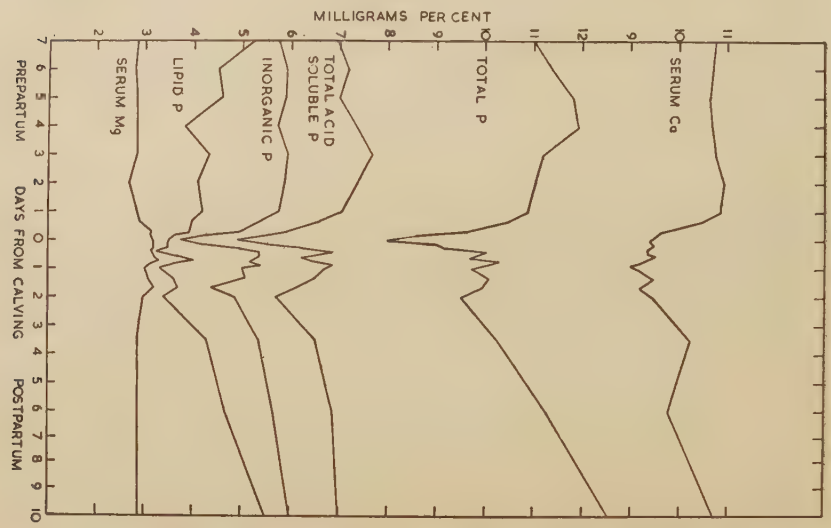


Fig. 1. Changes in blood constituents of normal parturient cows. The data for serum calcium, serum magnesium and plasma inorganic phosphate were obtained from observations on 32 cows and the other phosphate fractions from 18 cows.

It will be seen that the serum calcium remained level until two days before calving, after which its value dropped by about 2 mg. per cent to a low point within 24 hours post-partum. This was followed by a gradual increase until 10 days after calving. The serum magnesium remained very constant until about 12 hours before calving. From then until about two days after calving it had a slightly higher level, the highest mean being recorded in the first day. From two days after calving its level was comparable to the pre-calving level.

The plasma inorganic, total acid soluble and total phosphates showed close similarity in their changes. The levels remained fairly constant until two days pre-partum after which they began to fall markedly. As parturition approached, this decline became increasingly rapid and the lowest values were recorded at parturition. After calving there was an immediate reversal of the prepartum trends and very rapid increases were observed within a few hours of calving. The levels did not alter much for the next 24 hours but thereafter secondary, though smaller, reductions in level were observed. From the second day after calving the phosphate levels increased over the next few days to their pre-partum values. Lipid phosphate was relatively constant during the few days before calving and then showed a slight drop which persisted with fluctuations for a couple of days. Thereafter it increased throughout the period of observation.

Fig. 2

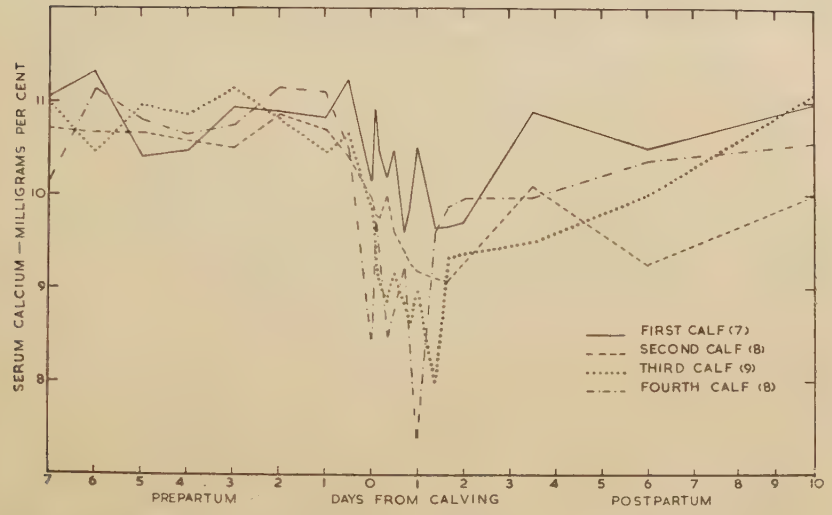


Fig. 2. Comparison of changes in serum calcium in normal parturient cows of different calving groups. Figures in parenthesis indicate the number of cows in each group. The fourth calf group includes cows at their fourth to sixth calving.

Fig. 3

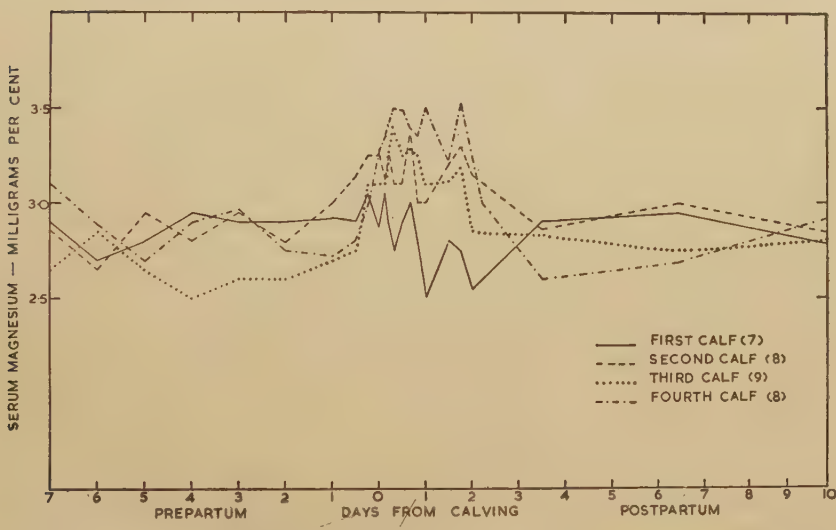


Fig. 3. Comparison of changes in serum magnesium in normal parturient cows of different calving groups. Figures in parenthesis indicate the number of cows in each group. The fourth calf group includes cows at their fourth to sixth calving.

Figures 2 to 4 show the mean levels for serum calcium and magnesium and plasma inorganic phosphate in the same cows as in Figure 1, divided into the four calving groups, throughout the

parturient period. The organic phosphate fractions are not shown as too few estimations were made in some of the groups.

Fig. 4

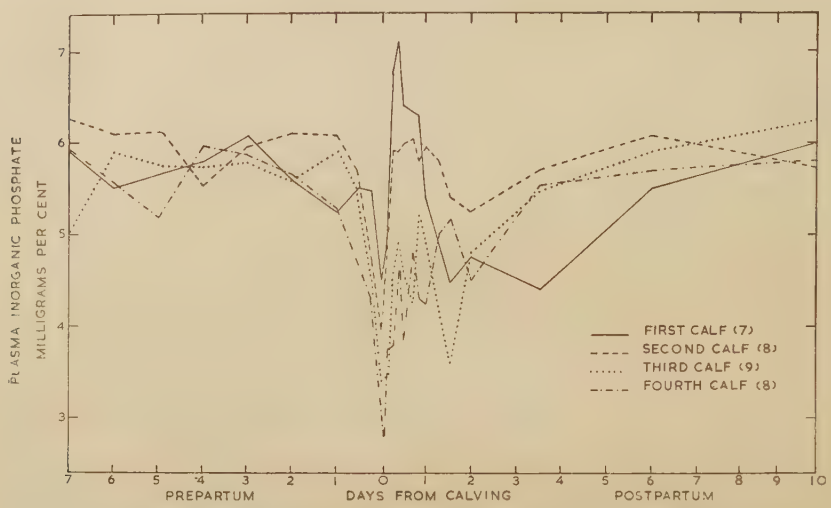


Fig. 4. Comparison of changes in plasma inorganic phosphate in normal parturient cows of different calving groups. Figures in parenthesis indicate the number of cows in each group. The fourth calf group includes cows at their fourth to sixth calving.

The figures show that in all three fractions the changes tended to become greater with each succeeding parturition. Thus from calving until 24 hours postpartum, the cows calving for the first time showed the highest mean serum calcium values (Fig. 2) while those at their fourth or subsequent calving had the lowest mean calcium levels, the other two groups occupying an intermediate position. Serum magnesium (Fig. 3) was similar in that the younger calving cows showed a smaller change than the older cows but it was eight hours after calving before the levels were arranged with the youngest cows lowest and the oldest cows highest. The inorganic phosphate (Fig. 4) showed very marked differences between calving groups; the youngest cows had the highest mean value at and after calving.

Detailed Analysis of Results

In determining the significance of the changes in the blood constituents and of the differences between calving groups some form of analysis of data was rendered necessary by the fact that it was impossible to obtain samples from all cows at exactly the same times relative to calving, and by the fact that the total bulk of the data was too unwieldy for straightforward statistical analysis. Two methods have been adopted: (1) Comparisons have been made between the levels of the blood fractions at a few definite times before, at and after parturition; (2) in analysing the differences between groups, animals

at their first and second parturitions have been classed together and compared with a second group in which animals at their third and subsequent parturition have been taken together.

Tables 1 to 3 show the results of analyses of variance of the data treated in this way. Table 1 shows the results from all cows which were blood sampled two days before, at, and four hours, 24 to 40 hours, two days and 10 to 20 days after calving. Table 2 shows the results of similar analyses of variance carried out where samples were obtained from cows two days, one day and 12 hours before calving and at calving. Table 3 shows similar data obtained at calving and four hours, 12 hours and 24 to 40 hours later. Thus, taking the three tables together, a fairly close study of changes from one time to another may be made.

Serum calcium. There was no evidence of a fall in the mean serum calcium level in the cows at their first and second calving until after four hours post-partum, when a fall of approximately 1 mg. per cent occurred. This low level persisted until two days after calving and was lower than the levels found at or prior to calving. The return of the serum calcium to normal was complete by 10 to 20 days after calving. Highly significant changes occurred in cows at their third to sixth calving during the parturient period. There was a fall of some 2 mg. per cent between two days before calving and calving, and about two-thirds of this fall occurred within the last 12 hours. The level remained significantly lower than the pre-partum values until at least two days post-partum. Return to normal levels was complete by 10 to 20 days after calving.

Serum magnesium. There was a slight increase in serum magnesium from two days before calving until four hours after calving in both calving groups, after which the levels gradually fell.

Plasma inorganic phosphate. Both groups showed a significant decrease from two days pre-partum until calving. Table 2 indicates that more than half of this fall occurred in the last 12 hours pre-partum. Parturition was followed by an immediate reversal of this trend in both groups. In the younger group there was a remarkable increase, the mean value of 6·63 mg. per cent at four hours post-partum (Table 1) being in excess of the precalving level. This level was maintained until about 12 hours after calving and was followed by a decrease until two days post-partum, when the value was significantly lower than the precalving value. Return to normal was complete by 10 to 20 days after calving. In the older cows there was only a gradual increase after calving throughout the period under observation. Although there was a significant increase by 12 hours post-partum the values from calving until two days after calving remained significantly lower than the prepartum value.

Plasma total acid soluble phosphate. The changes in this fraction very closely resembled those of inorganic phosphate. In the case of the first and second calving group the changes in Tables 1 and 2 were

TABLE 1

CHANGES IN SERUM CALCIUM AND MAGNESIUM AND PLASMA PHOSPHATES IN NORMAL PARTURIENT COWS

Fraction	*No. of Calving*	*No. of Cows*	*(Before) 2 days*	*Calving*	*Time from Calving 4 hours*	*24–40 hours*	*2 days*	*(After) 10–20 days*	*Mean*	*S.E.*	*Sig.*
Serum Calcium*	I & II	5	10·29	10·16	10·36	9·47	9·56	10·28	10·02	0·251	N.S.
	III–VI	9	11·12	9·32	9·18	9·32	9·85	10·68	9·91	0·258	0·01
Serum Magnesium*	I & II	5	2·96	3·15	3·27	2·86	2·98	2·89	3·02	0·139	N.S.
	III–VI	9	2·71	3·04	3·27	3·09	2·87	2·84	2·97	0·159	N.S.
Plasma Inorganic Phosphate†	I & II	5	5·98	4·68	6·63	6·10	4·93	5·73	5·68	0·351	0·01
	III–VI	9	5·52	3·32	4·24	4·28	4·29	6·18	4·64	0·305	0·01
Plasma Total Acid Soluble Phosphate†	I & II	3	7·60	5·78	7·47	7·00	5·81	6·38	6·67	0·486	N.S.
	III–VI	5	6·78	3·87	5·09	5·80	5·38	7·59	5·76	0·504	0·01
Plasma Lipid Phosphate†	I & II	3	5·77	4·83	4·73	5·70	5·38	7·30	5·62	0·405	0·05
	III–VI	5	3·42	3·26	2·94	2·86	3·26	4·68	3·40	0·405	N.S.
Plasma Total Phosphate †	I & II	3	12·53	10·32	12·13	12·65	10·83	13·03	11·92	0·563	0·05
	III–VI	5	10·36	7·08	7·94	8·54	8·82	12·88	9·27	0·369	0·01

* Ca. or Mg. mg. per 100 ml. Serum.
† Mg. P. per 100 ml. Plasma.

TABLE 2

CHANGES IN SERUM CALCIUM AND MAGNESIUM AND PLASMA PHOSPHATES IN NORMAL COWS BEFORE PARTURITION

Fraction	*No. of Calving*	*No. of Cows*	*Time before Calving*			*Calving*	*Mean*	*S.E.*	*Sig.*
			2 days	*1 day*	*12 hours*				
Serum Calcium*	I & II	7	10·84	11·04	11·01	10·67	10·89	0·125	N.S.
	III–VI	6	11·31	10·83	10·26	8·48	10·23	0·097	0·01
Serum Magnesium*	I & II	7	2·87	2·94	3·01	3·10	2·98	0·071	N.S.
	III–VI	6	2·77	2·90	2·96	3·36	2·99	0·099	0·01
Plasma Inorganic Phosphate†	I & II	7	6·03	5·94	5·54	4·46	5·49	0·229	0·01
	III–VI	6	5·68	5·24	4·89	3·22	4·76	0·258	0·01
Plasma Total Acid Soluble Phosphate†	I & II	3	7·60	7·20	6·98	5·78	6·89	0·434	N.S.
	III–VI	4	7·21	6·83	6·26	4·11	6·10	0·419	0·01
Plasma Lipid Phosphate†	I & II	3	5·77	5·53	5·18	4·83	5·33	0·393	N.S.
	III–VI	4	3·93	4·34	4·03	3·60	3·97	0·209	N.S.
Plasma Total Phosphate†	I & II	3	12·53	12·43	11·50	10·33	11·70	0·596	N.S.
	III–VI	4	11·40	10·94	10·60	7·50	10·11	0·230	0·01

* Ca. or Mg. mg. per 100 ml. Serum.
* Mg. P. per 100 ml. Plasma.

TABLE 3

CHANGES IN SERUM CALCIUM AND MAGNESIUM AND PLASMA PHOSPHATES IN NORMAL COWS AFTER PARTURITION

Fraction	*No. of Calving*	*No. of Cows*	*Calving*	*Time after Calving*			*Mean*	*S.E.*	*Sig.*
				4 hours	*12 hours*	*24-40 hours*			
Serum Calcium*	I & II	5	10·45	10·45	9·41	9·40	9·83	0·234	0·05
	III–VI	7	8·66	8·87	8·98	9·44	8·99	0·271	N.S.
Serum Magnesium*	I & II	5	3·24	3·36	3·13	2·94	3·17	0·077	0·05
	III–VI	7	3·16	3·40	3·38	3·25	3·30	0·114	N.S.
Plasma Inorganic Phosphate†	I & II	5	4·30	6·24	6·34	5·32	5·55	0·182	0·01
	III–VI	7	3·06	3·79	4·31	4·61	3·95	0·360	0·05
Plasma Total Acid Soluble Phosphate†	I & II	4	4·80	7·54	7·56	6·55	6·61	0·198	0·01
	III–VI	5	3·73	4·96	5·41	6·15	5·06	0·408	0·01
Plasma Lipid Phosphate†	I & II	4	3·53	3·38	3·68	4·26	3·71	0·189	0·05
	III–VI	5	3·26	2·52	3·04	2·48	2·92	0·360	N.S.
Plasma Total Phosphate†	I & II	4	8·93	10·83	11·93	11·21	10·73	0·384	0·01
	III–VI	5	6·94	7·68	8·54	8·96	8·03	0·382	0·01

* Ca. or Mg. mg. per 100 ml. Serum.
† Mg. P. per 100 ml. Plasma.

not significant, possibly because of the small number of cows under observation, but the increase from calving until four and 12 hours post-partum was highly significant (Table 3). The alterations in the total acid soluble phosphates of the older cows were all significant.

Plasma lipid phosphate. There was a slight fall in level until shortly after calving in both groups, but the only significant change occurred in the cows at their first and second calving, where an increase was noted at 10 to 20 days after calving.

Plasma total phosphate. The changes in this fraction were similar to those shown by inorganic phosphate.

Comparison of Calving Groups

Table 4 shows comparisons between the mean levels of the blood fractions in cows at their first and second calving and those at their third and subsequent calving, from two days before calving until 10 to 20 days post-partum. The number of samples entering into each mean and the S.E.s of the means are also shown. Significant differences between values are shown by asterisks.

The lowest recorded mean serum calcium value for the younger group of cows was 9·08 ± 0·273 mg. per cent at 40 hours after calving; that for the older group was 8·54 ± 0·323 mg. per cent at 20 hours after calving. The only point where a significant difference between the groups could be shown was eight hours post-partum.

The highest recorded mean serum magnesium level of cows at their first and second calving was 3·22 ± 0·137 mg. per cent at 16 hours post-partum, with a point four hours before calving which was almost as high. The highest mean value for cows at their third and subsequent calving was 3·46 ± 0·177 mg. per cent at eight hours post-partum. The only significant difference between the groups occurred at 5 to 7 days after calving.

The lowest inorganic phosphate means occurred at calving in both groups, the levels being 4·20 ± 0·280 mg. per cent and 3·15 ± 0·195 mg. per cent for the young and old cows respectively. Significant differences between the groups occurred at all points from calving until 24 hours post-partum.

The total acid soluble and total phosphates were similar to the inorganic phosphate. The lowest mean total acid soluble phosphate level in the younger group of cows was 5·56 mg. per cent one hour before calving, the value at calving being 5·66 mg. per cent. The lowest mean for the older cows was 3·80 mg. per cent. The lowest total phosphate means were 8·51 mg. per cent for the young cows one hour before calving and 6·80 mg. per cent for the old cows at calving. Significant differences between groups were observed at calving and at one, four, 12 and 20 hours post-partum in the case of total acid soluble phosphate and at two, four, eight and 16 hours after calving in the case of total phosphate. No significant differences between groups were observed in the case of lipid phosphate.

TABLE

SERUM CALCIUM AND MAGNESIUM AND PLASMA

Fraction	*No. of Calving*		*Days (Before) 2*	*1*	*12*	*4*	*1*	*0*	*2*
		No.	11	11	9	8	8	13	9
	I & II	Mean	10·87	10·78	10·86	10·17	9·94	10·13	10·19
Serum		S.E.	0·254	0·295	0·379	0·408	0·127	0·322	0·413
Calcium *		No.	16	11	9	4	8	13	12
	III-VI	Mean	11·05	10·85	10·54	9·94	9·81	9·15	9·61
		S.E.	0·163	0·260	0·305	0·424	0·422	0·365	0·369
		No.	11	11	9	7	8	13	9
	I & II	Mean	2·81	2·95	3·03	3·21	3·10	3·10	3·05
Serum		S.E.	0·124	0·096	0·027	0·087	0·151	0·110	0·074
Magnesium*		No.	16	11	9	4	8	14	12
	III-VI	Mean	2·66	2·70	3·76	3·08	2·91	3·17	3·13
		S.E.	0·090	0·146	0·137	0·184	0·229	0·085	0·192
		No.	11	11	9	9	9	13	9
	I & II	Mean	5·94	5·73	5·62	5·18	4·56	4·20	4·67
Plasma		S.E.	0·176	0·215	0·183	0·273	0·347	0·280	0·265
Inorganic Phosphate†		No.	17	11	9	4	8	14	12
	III-VI	Mean	5·62	5·59	5·06	4·45	3·69	3·15§	3·66§
		S.E.	0·236	0·270	0·225	0·175	0·408	0·195	0·230
		No.	6	7	4	5	7	8	6
	I & II	Mean	7·78	7·00	6·95	6·04	5·56	5·66	6·20
Plasma Total Acid		S.E.	0·241	0·202	0·121	0·391	0·335	0·584	0·647
Soluble Phosphate †		No.	8	7	5	2	3	7	6
	III-VI	Mean	6·81	7·09	6·29	5·50	4·72	3·80‡	4·66
		S.E.	0·609	0·568	0·353	0·200	1·566	0·314	0·471
		No.	6	7	4	5	7	9	6
	I & II	Mean	4·68	4·33	4·49	3·50	3·79	3·99	3·67
Plasma Lipid		S.E.	0·866	0·900	0·787	0·698	0·922	0·470	0·791
Phosphate†		No.	8	6	5	1	3	6	6
	III-VI	Mean	3·59	3·91	3·62	6·20	3·70	3·12	3·13
		S.E.	0·534	0·737	0·007	0·000	1·501	0·774	0·535
		No.	6	7	4	5	7	8	6
	I & II	Mean	12·02	10·98	11·09	9·50	8·51	9·00	10·18
Plasma Total†		S.E.	0·756	0·727	0·605	0·966	0·678	0·872	0·770
Phosphate		No.	8	7	5	2	3	7	6
	III-VI	Mean	10·35	10·66	10·10	10·15	8·53	6·80	7·72‡
		S.E.	0·680	0·752	0·883	1·950	1·934	0·615	0·710

* Ca. or Mg. mg. per 100 ml. Serum.
† Mg. P. per 100 ml. Plasma.
‡ Significantly different at 5% from corresponding I and II calving value.
§ Singificantly different at 1% from corresponding I and II calving value.

DISCUSSION

In general the results of this investigation confirm those of previous workers who have noted a fall in the levels of serum calcium and plasma phosphates and a rise in the level of serum magnesium in normal cows at parturition. The failure of some workers to

4

PHOSPHATE LEVELS IN NORMAL PARTURIENT COWS

Time from Calving (*After*)											
Hours								*Days*			
4	*8*	*12*	*16*	*20*	*24*	*32*	*40*	*2*	*3-4*	*5-7*	*10-20*
10	8	10	5	5	7	9	4	11	12	8	15
9·98	10·08	10·06	9·46	9·57	9·57	9·74	9·08	9·46	10·41	9·94	10·47
0·352	0·329	0·446	0·164	0·587	0·550	0·437	0·273	0·311	0·293	0·276	0·227
16	13	10	11	7	8	6	7	16	13	8	17
8·45	8·73‡	9·11	9·06	8·54	8·77	9·46	9·41	9·53	9·94	9·62	10·58
0·369	0·347	0·213	0·407	0·323	0·360	0·356	0·259	0·276	0·217	0·212	0·209
11	9	10	5	6	7	9	4	11	12	7	15
3·09	2·98	2·98	3·22	2·88	2·84	2·90	3·13	2·93	2·88	2·95	2·82
0·136	0·130	0·104	0·137	0·092	0·184	0·142	0·148	0·174	0·060	0·081	0·063
15	13	10	11	7	8	6	7	15	12	8	14
3·30	3·46	3·40	3·36	3·29	3·13	3·31	3·28	2·92	2·73	2·71‡	2·88
0·121	0·177	0·188	0·194	0·087	0·212	0·144	0·197	0·125	0·114	0·048	0·072
10	8	10	6	5	7	9	4	11	12	8	15
6·07	6·17	6·40	5·99	6·10	5·77	5·37	4·90	5·11	5·26	5·78	5·82
0·227	0·372	0·341	0·339	0·493	0·299	0·361	0·448	0·296	0·313	0·279	0·129
16	12	9	11	7	8	6	7	15	12	8	14
4·18§	4·75§	4·22§	4·54§	4·82‡	4·23§	4·72	4·09	4·65	5·48	5·32	6·08
0·333	0·400	0·414	0·293	0·345	0·298	0·378	0·367	0·289	0·302	0·301	0·262
8	6	6	5	4	4	5	4	8	6	6	8
7·24	7·55	7·33	6·59	7·74	6·96	6·74	6·19	6·07	6·33	6·93	6·71
0·265	0·376	0·414	0·679	0·130	0·599	0·477	0·231	0·368	0·496	0·245	0·199
8	6	6	6	4	1	6	3	8	7	5	6
5·21§	6·08	5·16§	6·37	5·88§	5·35	6·25	5·97	5·50	6·61	6·56	7·21
0·410	0·545	0·482	0·753	0·391	0·000	0·429	0·130	0·687	0·499	0·350	0·553
8	6	7	5	4	4	5	4	7	6	6	8
3·99	3·97	3·49	4·53	4·10	3·25	4·58	3·75	3·77	4·67	4·78	6·08
0·742	0·463	0·614	0·885	0·795	0·095	0·853	0·851	0·657	0·723	0·714	0·577
8	6	6	5	4	1	6	3	8	7	5	6
2·89	2·93	3·08	3·56	3·40	3·70	2·77	3·70	3·19	3·79	4·44	4·90
0·485	0·527	0·629	0·828	1·045	0·000	0·973	1·588	0·551	0·618	0·813	0·635
8	6	6	5	3	4	5	4	8	7	6	8
10·29	11·27	11·42	11·80	10·83	10·13	11·08	10·03	9·95	10·77	11·65	12·49
0·445	0·822	1·268	0·712	1·692	0·894	1·089	0·914	0·753	0·686	0·565	0·470
8	6	6	6	4	1	6	3	8	7	5	6
8·10‡	8·90‡	8·30	9·20‡	9·53	8·10	9·35	9·63	8·98	9·69	10·99	12·57
0·675	0·584	0·694	0·584	0·818	0·000	0·623	0·186	0·447	0·511	0·616	0·550

observe these changes was probably due to insufficient frequency of blood sampling about the time of parturition.

Inspection of Figure 2 shows there was a distinct tendency for the cows at their first and second calving to maintain higher serum calcium levels than older cows, but with the exception of eight hours post-partum there was no significant difference between their means

(Table 4). The fall in level which occurred in both groups took place 24 hours after calving in the first and second calving cows and a day earlier in older cows (Table 3). The serum calcium means in Tables 1 to 3, however, do not reflect the full magnitude of the changes as inspection of the data from individual cows showed that the lowest points occurred at varying times after calving. Calculation of means at specific times, therefore, masks the full extent of the changes. Accordingly, Table 5 has been prepared comparing the lowest values occurring up to one day before calving with the lowest values observed between 12 hours before and three days after calving. There was no material difference between the pre-partum serum calcium levels of the two age groups, but the lowest levels observed during the parturient period were significantly different. Significant reductions in serum calcium levels occurred in both age groups and the changes were significantly greater in older cows. This confirms the observations of Wilson and Hart (1932) regarding the increase in the fall in serum calcium level at parturition with advancing age. It also tends to support the contention that milk fever is a pathological exaggeration of a physiological process (Allcroft, 1947), as the incidence of the disease is much higher in cows which have had at least two previous parturitions.

Table 5 also shows the means of the highest observed pre-partum levels of serum magnesium compared with the highest observed parturient levels. This table shows no significant increase in the serum magnesium level of cows at their first and second calving but there was a significant increase in the older cows.

There were marked differences between the levels of inorganic, total acid soluble and total phosphates in the two calving groups from calving until about 24 hours afterwards; many of these differences are significant (Table 4). The levels reached their lowest recorded points at calving in both groups and most of the changes occurred in the last 12 hours pre-partum. The young cows made a very rapid recovery, as values of the same order as the initial levels were recorded within four hours of calving. The peak of this recovery appeared to be about 12 hours after calving, after which a secondary fall in level occurred, so that two days after calving the means were approximately the same as at calving. A study of individual cows indicated that the return to normal levels was gradual after this point. The changes immediately after calving in the older cows were different as there was no evidence of the rapid return to normal levels observed in the first and second calving cows. Instead, there was a gradual increase for the first two days after parturition, but even then the levels were still markedly lower than the pre-partum levels. At this stage, however, the mean level was not markedly lower than that for younger cows. As in the case of the younger cows, the recovery thereafter would seem to be gradual over the next few days.

Plasma lipid phosphate fell slightly to a low point about calving, but the change was not great. The most marked change occurred in

TABLE 5

COMPARISON OF SERUM CALCIUM AND MAGNESIUM LEVELS BEFORE AND DURING PARTURITION

1	2.	3	4			5			6	7		
Fraction	*No. of Calving*	*No. of Cows*	*Preparturient†*			*Parturient‡*			*Sig. of Change between 4 and 5*	*Change in Level*		
			Mean	*S.E.*	*Sig.*	*Mean*	*S.E.*	*Sig.*		*Mean*	*S.E.*	*Sig.*
Serum Calcium*	I & II	15	10·10	0·088	N.S.	9·18	0·284	0·05	0·01	— 0·92	0·266	0·01
	III–VI	17	10·20	0·157		8·11	0·277		0·01	— 2·09	0·275	
Serum Magnesium*	I & II	15	3·02	0·088	N.S.	3·23	0·086	0·05	N.S.	+ 0·21	0·094	0·01
	III–VI	17	2·92	0·097		3·57	0·111		0·01	+ 0·65	0·090	

* Ca. or Mg. mg. per 100 ml. serum.
† Lowest observed serum calcium or highest observed serum magnesium values up to one day before parturition.
‡ Lowest observed serum calcium or highest observed serum magnesium values from twelve hours until three days after parturition.

the younger cows 10 to 20 days after calving when a well-defined increase in level took place, but it would seem that this fraction had little effect on the levels of the other phosphate fractions.

One purpose of this investigation was to determine if the reduction in plasma inorganic phosphate represented a shift of phosphate from one fraction to another or whether it was part of a general fall in plasma phosphate level. The data presented show that the fall in plasma inorganic phosphate is not accompanied by an increase in any other plasma phosphate fraction, but that it is associated with a reduction in total acid soluble phosphate and hence in total phosphate. A few observations we have made on whole blood indicate that the whole blood total phosphate level also falls at parturition. In view of indirect evidence that there is no haemodilution at parturition, as evidenced by blood protein levels (Wilson and Hart, 1932) and the tendency for serum magnesium to move in the opposite direction, it is probable that the changes in blood phosphate levels are a reflection of an alteration in blood phosphate supply or demand.

In regard to the normal parturient changes to milk fever, Allcroft and Godden (1934) suggested a relationship between the changes in the blood ions of normal parturient cows and those of cows affected with milk fever. Verification of their hypothesis would be of value in guiding investigations into the aetiology of milk fever and there are several features of our results which support its acceptance. Probably the most important is the observation that the changes in blood calcium, magnesium and phosphates become progressively greater with advancing age—the relative positions of the calcium and phosphate fractions in the different groups at and shortly after calving are quite striking. Moreover, it is significant that these changes occur within three days of calving, which is the period during which the majority of clinical cases of milk fever occurs.

If the hypothesis that parturient paresis is an exaggeration of normal changes at parturition is correct, one would expect to encounter subclinical cases of the condition. It may be of interest therefore to record that two cows showed dullness and uneasiness after calving and gave an impression of incipient parturient paresis but failed to develop typical clinical signs. In these the serum calcium fell to 5·42 mg. per cent and 6·00 mg. per cent respectively, the plasma inorganic phosphate to 1·70 mg. per cent and 2·75 mg. per cent, while the other phosphate fractions followed similar trends; the serum magnesium rose to 4·10 mg. per cent and 6·00 mg. per cent. These values are similar to many that have been obtained in clinical cases of milk fever. Van Soest and Blosser (1954) have also observed "borderline" cases of milk fever.

There is little doubt about the importance of the changes in serum calcium in milk fever, but the part played by changes in the blood phosphate is not so clear. In this connection it should be noted that the general levels of the inorganic, total acid soluble and total phosphates in older cows remain below normal for at least 24 hours

after calving, which is the period when a high incidence of milk fever is observed; during this period the phosphates of the younger cows are well within the normal range. Moreover, the very rapid recovery of the phosphates in the younger cows suggests that they have some mobilising mechanism which older cows lose, or alternatively, the younger cows have phosphate reserves not possessed by the older cows.

CONCLUSIONS

A detailed study has been made of the changes in serum calcium and magnesium and in plasma inorganic, total acid soluble, lipid and total phosphates in 32 normal parturient cows. Seven of these cows were having their first calf, eight their second, nine their third, and eight their fourth or subsequent calf.

Serum calcium levels fell at or within two days of calving to reach their lowest point about one day after calving; the fall was greater in older cows and the levels they dropped to were significantly lower than in the younger group.

Serum magnesium levels increased to reach their highest point about one day after calving; the rise was greater in older cows and the levels reached by older cows were significantly higher than in the younger group.

Inorganic, total acid soluble and total phosphates all decreased during the last 24 hours prepartum to reach their lowest point at calving; the fall was greater in older cows and the levels they dropped to were significantly lower than in the younger group. Calving was followed by an immediate rise in these plasma phosphate levels; the rise was more rapid in the younger group of cows. There was a slight secondary fall in these phosphate fractions about two days after calving and then a gradual return to prepartum levels.

Lipid phosphate showed only slight alteration at parturition and there were no differences in lipid phosphate levels between the groups.

The relation of these blood changes in normal parturient cows to those in milk fever is discussed.

ACKNOWLEDGMENTS

Our thanks are due to Dr. A. D. Buchanan-Smith and his farm staff for facilities in obtaining blood samples and to Dr. H. Paver for advice on the statistical analysis. We wish also to record our indebtedness to some members of the staff of the Royal (Dick) School of Veterinary Studies for technical assistance.

REFERENCES

Allcroft, W. M. (1947). *Vet. J.*, **103**, 2.
Allcroft, W. M., and Godden, W. (1934). *Biochem. J.*, **28**, 1004.
Barker, J. R. (1948). *Vet. Rec.*, **60**, 351.
Blood, D. C., and White, I. G. (1949). *Aust. vet. J.*, **25**, 114.

Clark, E. P., and Collip, J. B. (1925). *J. biol. Chem.*, **63**, 461.
Denis, W. (1922). *Ibid.*, **52**, 411.
Dryerre, H., and Greig, J. R. (1928). *Vet. Rec.*, **40**, 721.
Duckworth, J., and Godden, W. (1940). *J. dairy Res.*, **11**, 9.
Fish, P. A. (1929). *Cornell Vet.*, **19**, 147.
Fish, P. A. (1930). *11th Int. Vet. Congr. London.*, **2**, 330.
Fiske, C. H., and Subbarow, Y. (1925). *J. biol. Chem.*, **66**, 375.
Godden, W., and Allcroft, W. M. (1932). *Biochem. J.*, **26**, 1640.
Goulden, C. H. (1936). *Methods of Statistical Analysis*, Burgess Publ. Co.; Minneapolis, Minn.
Greenhill, A. W., and Pollard, J. (1935). *J. Soc. chem. Ind.*, **54**, 404.
Hawk, P. B. (1947). *Practical Physiological Chemistry*, 12th Edit., J. & A. Churchill; Lond.
Hayden, C. E. (1938). *Cornell Vet.*, **28**, 152.
Little, W. L., and Mattick, E. C. V. (1933a). *Vet. Rec.*, **45**, 238; (1933b). *Ibid.*, 1091.
Little, W. L., and Wright, W. C. (1925). *Ibid.*, **37**, 631.
Palmer, L. S., Cunningham, H. S., and Eckles, C. H. (1930). *J. dairy Sci.*, **13**, 174.
Robertson, A. (1949). *Vet. Rec.*, **61**, 333.
Robertson, A., Burgess, J. W., Marr, A., and Milne, B.J.C. (1948). *Ibid.*, **60**, 505.
Seekles, L., Sjollema, B. and Van der Kaay F. C. (1932). *Biochem. Z.* **244**, 424.
Van Soest, P. J., and Blosser, T. H. (1954). *J. dairy Sci.*, **37**, 185.
Wilson, L. T., and Hart, E. B. (1932). *Ibid.*, **15**, 116.

[*Received for publication, June 6th,* 1954.]

J. Comp. Path. 1955. Vol. 65.

OBSERVATIONS ON EXPERIMENTAL FOWL TYPHOID

By

H. Williams Smith

Animal Health Trust, Houghton Grange, Huntingdon

INTRODUCTION

Fowl typhoid has been produced in chickens by the oral administration of cultures of *Salmonella gallinarum* (Gordeuk, Glantz, Callenbach and Thorp, 1949; Hall, MacDonald and Legenhausen, 1949; Wilson, 1946) but the disease that results has not been closely studied. It seemed essential, therefore, to carry out such a study before the value of chemo-therapeutic agents and other methods of control could be accurately assessed. Many of the observations reported in this paper are of experiments designed primarily for that purpose. Others were made incidentally during an investigation of the defence-mechanisms of the alimentary tract of the chicken against bacterial invasion (Smith, 1954a) or during experiments conducted later on the chemotherapy of the disease (Smith, 1955).

MATERIALS AND METHODS

Chickens. Sex-linked Brown Leghorn x Barred Rock chickens from a Salmonella-free flock were used. Unless otherwise stated, they were cockerels, approximately 9 weeks old when they were put under experiment.

Diet. The chickens were maintained on a mash of the following composition given *ad lib.*

Fish meal, 10.0%; Decorticated ground nut meal, 5.0%; Dried yeast, 2.5%; Maize meal, 10.0%; Wheat meal, 15.0%; Barley meal, 17.0%; Oats, 15.0%; Middlings, 19.0%; Dried grass meal, 5.0%; Minerals, 1.5%.

Inoculum. One strain of *Salm. gallinarum,* No. 9, was employed in all experiments. It was maintained throughout on Dorset egg medium at 4°C. When required for use, it was sub-cultured into nutrient broth, and incubated at 37°C for 18 hours. Freeze-dried specimens of strain 9 prepared at the commencement of the work were compared with the Dorset egg culture from time to time; no variation in the virulence of the latter was noted.

Method of producing infection. Except in the case of the chickens used in the defence-mechanism experiments, which were infected by giving different amounts of strain 9 mixed with different food materials, infection was produced by the following method. Chickens were fasted for 18 hours and then given 0.1 ml. of an 18 hour broth culture of strain 9, standardized on every occasion to the same nephelometer reading, in 1 ml. of distilled water containing approximately 0.3 g. of alkali, the purpose of which was to neutralize the bactericidal action of the gastric juice in the gizzard. This amount of culture was shown by the method of Miles and Misra (1938) to contain 50 million viable bacteria. It was deposited in the oesophagus by means of a Pasteur pipette, care being taken to avoid contaminating the epiglottis.

Alkali. The alkali employed was a powder of the following composition. Powdered chalk, 40%; Colloidal kaolin, 43%; Magnesium trisilicate, 17%.

The examination of faeces for Salm. gallinarum. Cloacal swabs, taken so as to include a liberal portion of faeces, were usually used for this purpose. They were incubated at 37°C for 24 hours in selenite F medium (Hobbs and Allison, 1945) from which sub-cultures were made on to plates of desoxycholate-citrate-agar (D.C.S.S.) containing 1 per cent each of sucrose and salicin (Smith and Buxton, 1951). The plates were incubated at 37°C for 24 hours, and examined for the presence of *Salm. gallinarum.* In some cases the swabs were also inoculated directly on to D.C.S.S. medium.

Tracing the route of Salm. gallinarum *infection.* In these experiments, groups of 10 chickens were infected in the usual way and then killed after different intervals of time. Selected organs were carefully removed from the dead chickens, ground in sterile sand where necessary and added to 150 ml. of selenite-F medium which was then examined for the presence of *Salm. gallinarum* in the manner already described. The whole of the spleen, heart, pancreas, pharynx, one kidney, one lung, one femur and approximately one-half of the liver were cultured. Several grams of faeces, 1 to 2 ml. of blood and bile, the contents of the distal limb of the duodenum and the next few inches of the ileum, and a portion, about 3 in. long, taken from the middle of this section of the alimentary tract were also examined. Unlike the other materials, bile was incubated in nutrient broth instead of selenite-F medium. The pharynx and the portion of intestine were slit longitudinally after removal, washed in water, placed in ethyl alcohol for 1 min., and then re-washed in water before being ground.

Blood culture. With sterile precautions approximately 0.3 ml. of blood was removed from one of the wing veins by means of a syringe. Of this 0.1 ml. was cultured directly on to a plate of D.C.S.S. medium. The remainder was first incubated in the selenite-F medium and then cultured on to D.C.S.S. medium.

Agglutination tests. The plate agglutination test (Gordon and Brander, 1952) using whole blood and a stained antigen prepared from strain 9 was used throughout.

RESULTS

The Mortality Rate in the Experimental Disease

The survival times of 300 9 week-old cockerels given 50 million *Salm. gallinarum* bacteria in alkaline medium by mouth are illustrated in Fig. 1. All became infected and 182 (60.7 per cent.) died. Deaths were first observed on the 7th day of infection. The death rate then increased rapidly reaching a peak on the 10th day when 33 chickens died. It then decreased slowly until the 19th day when the decline became much more rapid, only 3 chickens dying after 21 days.

The Effect of Age and Sex on Resistance to Fatal Infection.

The effect of giving groups of 30 chickens of different age and sex 50 million *Salm. gallinarum* bacteria in alkaline medium by mouth

Fig. 1

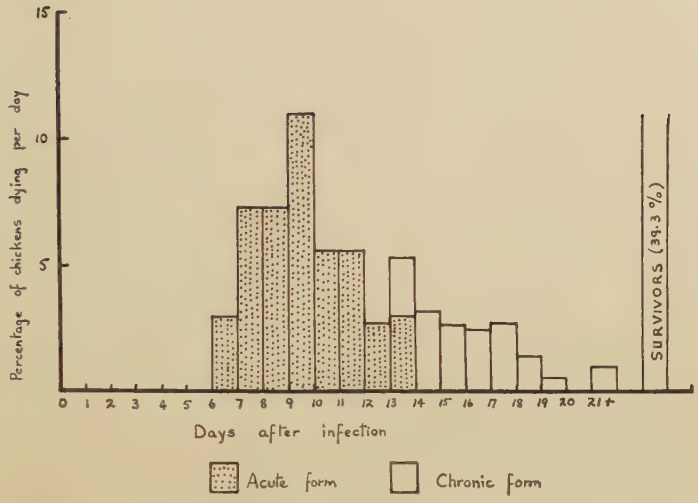

15
10
5
Percentage of chickens dying per day
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21+
Days after infection
SURVIVORS (39.3 %)
Acute form
Chronic form

FIG. 1. The survival times of 300 cockerels infected when nine weeks old

is shown in Table 1. One-day-old chicks were the most susceptible. A higher degree of resistance was exhibited by 3-day-old chicks, an observation that was confirmed in other experiments in which much larger numbers of chicks of both ages were used. Little difference was noted between the resistance of 17-day-old and 3-day-old but the results for 4, 9 and 14-week-old chickens showed a progressive increase of resistance with age as indicated by the longer survival times and lower death rates. All these results were obtained when male chickens were used. No difference was noted between the results of 9-week-old female chickens and those for male chickens of the same age, an observation that was confirmed in another experiment involving 62 9-week old females and a similar number of males of the same age, the number of survivors in each group being 19 and 20 respectively. Quite different results were obtained when mature chickens of either sex were used. They were very susceptible to fatal infection, their resistance resembling that of 3-day-old chickens.

The effect of giving groups of 30 9-week-old and 1-day-old chickens different doses of *Salm. gallinarum* culture by mouth is shown in Table 2. Much smaller numbers of *Salm. gallinarum* produced infection in 1-day-old than in 9-week-old chickens. As in the previous experiment, death occurred much more commonly as a sequel to infection in the 1-day-old chickens than in the 9-week-old chickens. In both age groups no marked alteration in the morbidity/mortality ratio was noted when different dose levels of *Salm. gallinarum* were compared. Very little difference, too, was noted in the course of the disease, including survival times, given different numbers of bacteria.

TABLE 1

THE FREQUENCY DISTRIBUTION OF THE SURVIVAL TIMES OF CHICKENS IN GROUPS OF 30 OF DIFFERENT AGE AND SEX

Age	*Sex*	*Days after infection*															*Number that survived*
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17+	
1 day	male	6	16	8	0	0	0	0	0	0	0	0	0	0	0	0	0
3 „	„	0	1	2	10	7	2	3	2	0	0	1	2	0	0	0	0
17 „	„	0	1	5	8	7	3	1	0	2	0	1	0	0	0	0	2
4 weeks	„	0	0	1	3	8	4	2	3	1	1	0	0	0	0	0	7
9 „	„	0	0	0	0	0	3	3	4	2	1	2	2	0	1	1	11
9 „	female	0	0	0	0	1	3	3	5	1	1	2	0	2	1	1	10
14 „	male	0	0	0	0	0	0	1	4	2	2	3	0	0	0	2	16
24 „	female (in lay)	0	0	2	10	7	1	2	1	0	1	1	0	1	0	0	4
64 „	female (at end of lay)	0	0	2	7	6	5	4	0	1	2	0	0	1	0	2	0
64 „	male	0	0	1	6	11	4	3	2	0	1	1	1	0	0	0	0

TABLE 2

THE SUSCEPTIBILITY OF ONE-DAY-OLD AND 9-WEEK-OLD CHICKENS TO *Salm. gallinarum* INFECTION

Number of Salm. gallinarum *bacteria administered (in millions)*	*One-day-old*		*9-week-old*	
	No. of 30 *that*		*No. of* 30 *that*	
	Became infected	*Died*	*Became infected*	*Died*
50.0	30	30	30	21
5.0	30	30	27	14
0.5	30	28	12	5
0.05	30	26	0	0
0.005	1	1	0	0

The bacteria were administered by mouth in alkaline medium. The 9-week-old chickens were first fasted for 18 hours; this was not necessary in the case of the day-old-chickens.

The Clinical Features of the Experimental Disease

Signs of ill-health which were first noted in chickens 3 to 4 days after the administration of *Salm. gallinarum*, became progressively more apparent. The infected chickens appeared listless and reluctant to move about. They stood motionless for long periods of time with the feathers puffed out, the head sunk on to the chest and with one or both eyes closed. Diarrhoea was apparent 5 to 6 days after infection, the faeces being mucoid and yellow in colour. Deaths started in groups of infected chickens within a few days of the appearance of diarrhoea. The disease became chronic in the case of chickens that did not die in the acute stage of the disease. Such chickens showed progressive emaciation and intense anaemia until death or slow recovery resulted.

The rectal temperatures, and food consumption of 2 infected chickens together with the results of examining their blood and faeces for *Salm. gallinarum* and their blood for agglutinins to this organism are shown in Figs. 2 and 3. They were selected from a group of 12 as typical examples of chickens that died either in the acute or chronic phase of the disease. Temperatures were recorded twice daily and the other estimations, except the first faecal examination which was made 6 hours after the commencement of the experiment, were made once daily. These examinations were carried out for 25 days.

Of the 12 chickens, 6 died in the acute phase of the disease, 1 on the 6th, 2 on the 8th, and 3 on the 9th day after infection. The disease became chronic in the remaining 6 chickens, 2 of which died, 1 on the 18th and 1 on the 20th day.

Fig. 2

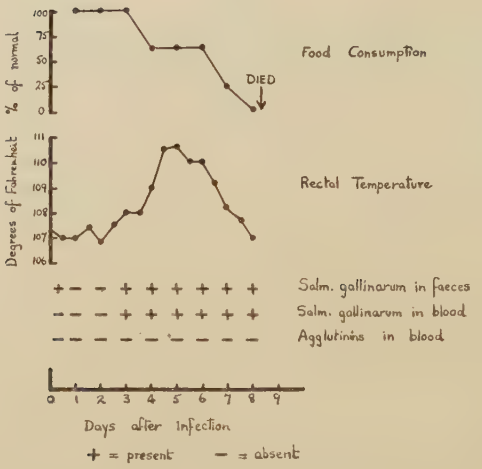


FIG. 2. Chicken that died in the acute phase

Fig. 3

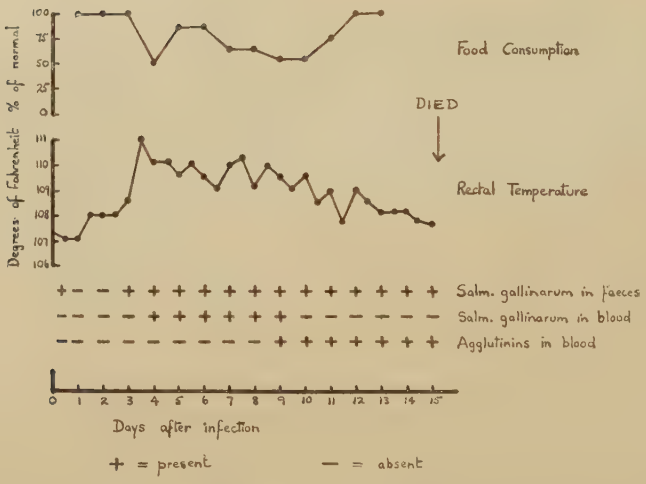

% of normal
100
75
50
25
0
Food Consumption
DIED
Degrees of Fahrenheit
111
110
109
108
107
106
Rectal Temperature
+ − − + + + + + + + + + + + Salm. gallinarum in faeces
− − − + + + + + − − − − − − Salm. gallinarum in blood
− − − − − − − − + + + + + + Agglutinins in blood
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days after infection
+ = present
− = absent

FIG. 3. Chicken that died in the chronic phase

Salm. gallinarum was recovered from the faeces of all 12 chickens 6 hours after it was administered. Examinations 1 day after infection were completely negative. One chicken became positive again after 2 days, 4 after 3 days and 4 after 4 days. *Salm. gallinarum* was not isolated again from the remaining 3 chickens until 6, 7 and 9 days after infection. All chickens were then consistently faecal-positive until they died or until the experiment was completed. *Salm. gallinarum* was not present in large numbers in the faeces except

immediately before death in the case of chickens that died in the acute phase of the disease. For example, it was only isolated by direct culture 12 times during the examination of 44 consecutive specimens shown to be positive by selenite enrichment.

Salm. gallinarum was never found in the blood of the chickens within 2 days of infection. It was first found in 1 chicken at 2 days, 6 at 3 days, 4 at 4 days and the remaining one at 5 days after infection Following its initial isolation, *Salm. gallinarum* was recovered at each daily examination from the blood of all the chickens that died in the acute phase of the disease. Bacteraemia was present in the other 6 chickens for a period of 4 to 7 days only. It was never present in large numbers in the circulating blood of any of the 12 chickens except immediately before death in those that died in the acute phase when counts in the region of 15,000 to 200,000 per ml. of blood were recorded. At other times never more than 50 bacteria per ml. were found; at 14 of 20 examinations *Salm. gallinarum* was isolated only by the use of selenite medium.

The rectal temperatures of the chickens remained around the normal figure of 107°C for the first 2 days after infection. Higher temperatures, in the region of 110 to 111°C, were then recorded, the increase coinciding with the presence of *Salm. gallinarum* in the circulating blood. These temperatures persisted in the case of the chickens that died in the acute phase until shortly before death. In the others, high temperatures were found throughout the bacteraemic phase. The body temperature then commenced to decrease but in the case of most of the survivors it was still 1 to 2° above normal when the experiment was concluded.

By means of the rapid whole blood plate test, agglutinins to *Salm. gallinarum* were never found in the blood of the 6 chickens that died in the acute phase of the disease. They were first found in the other 6 chickens 8 to 9 days after infection. By the 12th day maximum titres of 1/25 to 1/50 were recorded, titres which increased very little during the remainder of the observation period.

Food consumption was normal for the first 3 or 4 days after infection. It then dropped to one half to two thirds of normal. In the case of the chickens that died in the acute phase it usually dropped further to about one third of normal and in the day before death little or no food was consumed. The food consumption of the other chickens did not fall below one half of normal. It rose to about two thirds of normal on the 7th day and became normal after the 9th day.

Pathology

The experimental disease could be classified on a pathological basis into 2 types, the acute and the chronic.

The acute type. Very little loss in body weight occurred in chickens dying in the acute phase of the disease. The feathers around the vent were usually covered with liquid greenish-yellow faeces. The skeletal

muscles were diffusely congested. The main changes were noted in the liver which was enlarged, congested, friable and dark red in colour. Occasionally the liver was bronze-coloured. Microscopical examination revealed the liver to be diffusely infiltrated with large cells, probably stem cells. The spleen was also very enlarged. A catarrhal inflammation was present throughout most of the intestine, which contained a slimy green exudate.

The chronic type. Chickens dying in the chronic phase of the disease were very emaciated and intensely anaemic. A proliferative cellular reaction was present in several organs, those most commonly affected being the heart, intestine, pancreas and liver. In the heart this reaction took the form of white nodular masses scattered throughout the myocardium and varying in size from that of a millet seed to that of a pea (Plate 1). These lesions bulged out from the surface of the heart, distending its contour. In some cases they accounted for half the volume of the heart. They were definitely cellular in character and consisted mainly of stem or reticular cells but lymphocytes, monocytes, polymorphs and fibroblasts were also present. No obvious necrosis of the muscle fibres was noted, neither was it seen in the hearts of infected chickens that died or were killed before visible lesions would normally be present. The lesions described above were most noticeable 17 to 24 days after infection. At 28 days the white nodular masses were much less obvious and were definitely regressing. From this time onwards the most noticeable heart lesion was a pericarditis of varying severity. A considerable amount of yellow opaque fluid was present in the pericardial sac and the whole surface of the heart was covered with a comparatively thick layer of fibrin .Cauliflower-like deposits of yellow fibrinous-like material, loosely attached to the outer surface of the heart, were sometimes noted. A considerable degree of compensatory hypertrophy was present in many severely affected hearts. Heart lesions were often present in chickens killed 60 and even 80 days after infection although resolution was often advanced; only a slight amount of pericardial fluid was present. the heart was less enlarged and a fine layer of fibrin, barely noticeable, extended over its surface.

Intestinal lesions were most obvious 16 to 24 days after infection. They occurred as flat white irregular areas from about 4 sq. mm. to 1 sq. cm. in size (Plate 2) scattered mainly throughout the distal loop of the duodenum and the first 8 inches or so of the ileum. They were much less commonly found in the lower parts of the alimentary canal. Although they mainly involved the inner layers of the intestine they could be clearly seen through its external surface, the tissue between the lesions being intensely inflamed. The mucous membrane over many lesions had eroded and ulcers resulted. These were covered with whitish-yellow mucoid material which, when touched, came away easily from the underlying tissue in which several small haemorrhagic areas were present. The mucous membrane of the

intestine was very inflamed and the intestinal contents consisted mainly of a greenish-yellow catarrhal fluid. The lesions were wholly cellular in structure consisting mainly of mononuclear immature cells. They resolved comparatively rapidly in chickens that survived. At 35 days or more after infection they were very small and barely discernible from the surrounding tissue.

Lesions were most noticeable in the pancreas at 16 to 24 days after infection and were rarely seen at 35 days. They were round, circumscribed and 2 to 4 mm. in diameter (Plate 2). Never more than six were found in any chicken. Histologically, they resembled the early heart lesions.

The infiltrating cells found in the liver in the acute phase were often more numerous in the chronic phase and very occasionally they were aggregated together in sufficient numbers to be visible to the naked eye as small white spots scattered irregularly over the surface of the liver.

The Distribution of Lesions in Infected Chickens

A post-mortem examination carried out on the 182 9-week-old chickens referred to previously in the section on the mortality rate (Fig. 1) revealed that 135 (74.2 per cent.) had died in the acute phase and that the remaining 47 (25.8 per cent.)fatal cases possessed lesions typical of the chronic form of the disease. These lesions were never found in chickens dying before 14 days after infection and of the 16 dying on that day 9 possessed these lesions and 7 did not. They were present in all chickens dying after the 15th day. When the heart and intestines only were considered, 39 of the 47 chickens that died of the chronic form of the disease possessed lesions in both organs, 6 in the heart only and 2 in the intestine only. Lesions were also present in the pancreas of 9 and in the liver of 2 of these chickens.

TABLE 3

THE DISTRIBUTION OF LESIONS IN CHICKENS THAT DIED FROM, OR WOULD HAVE RECOVERED FROM, THE CHRONIC FORM

Category	*Number examined*	*Percentage of chickens with lesions in*					
		Heart and intestines	*Intestine only*	*Heart only*	*Pancreas*	*Liver*	*None*
Fatal	71	84.5	5.6	9.9	16.9	2.8	0
Non-fatal	147	18.4	5.4	73.4	12.9	8.1	2.8

The chickens classified as "fatal" succumbed to the chronic phase of the disease within 21 days of infection. The "non-fatal" chickens were killed at 21 days and were classified as such because the mortality rate after this time was always very low.

The distribution of lesions in 71 chickens that died of the chronic form of the disease and in a similar group of 147 infected chickens that were killed 21 days after infection for diagnostic purposes and which, in all probability, would have recovered are compared in Table 3. Intestinal lesions were found much more commonly in the chickens that died than in those that lived. Heart lesions were found to almost an equal extent in both groups of chickens. The lesions were also equally severe in both groups and it was difficult to understand why many chickens possessed such extensive lesions and yet, when alive, were not obviously ailing. There was very little difference between the frequency of pancreatic and liver lesions in each group. No visible lesions were found in only 6 (2.8 per cent.) of the chickens in the non-fatal group; infection was diagnosed in these by the demonstration of blood agglutinins and/or *Salm. gallinarum* in the faeces.

Pathogenesis

The result of culturing from different sites in groups of 10 chickens at different intervals of time after the administration of *Salm. gallinarum* by mouth is shown in Table 4. *Salm. gallinarum* was found in the liver, spleen or lung of 3 of the 10 chickens killed 3 hours after administration and was recovered with increasing frequency from the spleen and liver at 6, 12, 24 and 48 hours. The reverse was true in the case of lung culture, completely negative results being obtained at 48 hours. *Salm. gallinarum* was isolated from both the intestinal wall and intestinal contents of all 10 chickens killed at 3 hours and 6 hours. The number of positive isolations then decreased in the case of the intestinal contents, completely negative results being obtained at 24 and 48 hours. *Salm. gallinarum,* however, was found in the intestinal wall of 5 of the chickens examined at 24 hours and 6 of those examined at 48 hours. It was not found in the heart wall or the femur.

Since it had been shown previously that a bacteraemic phase usually commenced on the 3rd day after administration and lasted for several days the next group of chickens were examined on the 20th day and further groups on the 35th, 54th and 83rd days. *Salm. gallinarum* was isolated from one or more organs of all 10 chickens at 20 days. The number of positive isolations then declined at each successive examination until it was only recovered from 5 of the 10 chickens killed at 83 days. Contrary to the results obtained before the bacteraemic phase, *Salm. gallinarum* was found more frequently in the heart wall than in any other organ. It was fairly commonly isolated from the liver, spleen and pancreas, intestinal wall and intestinal contents but only rarely from the lung, femur and the bile. A fairly close correlation existed between the results of culturing the intestinal wall and the intestinal contents.

TABLE 4

THE ROUTE OF *SALM. GALLINARUM* INFECTION

Time after administration	*No. of 10 chickens from which* Salm. gallinarum *was recovered from*									
	Lung	*Heart*	*Femur*	*Pancreas*	*Spleen*	*Liver*	*Bile*	*Intestinal wall*	*Intestinal contents*	*Any site except the intestinal tract*
3 hr.	2	—	—	—	1	2	—	10	10	3
6 hr.	3	—	—	—	3	2	—	10	10	4
12 hr.	2	0	0	—	3	5	—	5	2	6
24 hr.	1	0	0	—	5	6	—	5	0	8
48 hr.	0	—	—	—	7	8	—	6	0	10
					BACTERAEMIC PHASE					
20 days	3	9(10)	2	4(2)	3	4(2)	1	6(4)	6	10
35 days	2	8(9)	0	1	3	4	2	3(1)	4	9
54 days	0	5(9)	0	2	2	2	0	2(1)	2	6
83 days	1	5(5)	1	2	2	3	0	1	1	5

The figures in brackets indicate the number of chickens with macroscopic lesions in the relevant organs. *Salm. gallinarum* was never isolated from the blood of any of these 90 chickens.

Blood culture of all the 90 chickens used in this experiment performed immediately they were killed yielded completely negative results as did blood in 1 ml. amounts taken from 10 of the chickens at hourly intervals for the first 6 hours after infection. Similarly, *Salm. gallinarum* was not recovered from the kidneys of any of the 10 chickens killed at 35 days, the only chickens in which these organs were examined.

It was originally planned to infect the chickens used in this experiment by giving them food to which *Salm. gallinarum* bacteria had been added so that the anterior portion of the alimentary tract would also be examined. Experiments, however, showed (Smith, 1954b) that some of the *Salm gallinarum* bacteria administered in this way were inhaled into the lower respiratory tract and, consequently, were recovered from a high proportion of lungs. In these experiments the distribution of the bacteria in organs other than the lungs was similar to that shown in Table 4. Positive cultures were also obtained from the pharynx of 5 of the 10 chickens killed at 3 hours, 2 of those killed at 6 hours and none of those killed at 24 and 48 hours. Experiments showed that these positive results were probably due to contamination rather than penetration of the pharyngeal wall.

In all examinations up to and including those made at 48 hours, the numbers of *Salm. gallinarum* bacteria present in sites other than the intestinal contents were very small since they were never recovered by direct culture techniques. Larger numbers were present immediately after the bacteraemic phase. They then declined rapidly. For example, in another experiment, *Salm. gallinarum* was only isolated by direct culture from the heart wall of 2 of the 20 chickens that had been infected 35 days previously, despite the fact that they all contained severe lesions; they were all positive following enrichment in selenite medium. It will also be seen from Table 4 that negative results sometimes followed even selenite enrichment of cultures prepared from the heart, pancreas, liver and intestinal wall which contained obvious lesions of *Salm. gallinarum* infection.

TABLE 5

THE RELATIONSHIP BETWEEN THE RESULTS OF POST-MORTEM EXAMINATION AGGLUTINATION TESTS AND FAECAL CULTURE IN CHICKENS GIVEN VARYING DOSES OF *Salm gallinarum*

Classification	*No. of chickens*	*No. that were*		
		Agglutination +	*Faeces* +	*Lesions* +
Infected	218	218	191	212
Non-infected	702	0	0	0

The examinations were carried out 20 days after giving the bacteria by mouth.

The Relationship between the Results of Post-mortem Examination, Agglutination Tests and Faecal Culture

The results of submitting 920 chickens to agglutination tests, faecal culture and post-mortem examination 20 days after they were given different amounts of *Salm. gallinarum* culture by mouth is shown in Table 5. Of these, 702 were negative to all 3 tests, and, consequently, were classified as non-infected. The remaining 218 were positive to the agglutination test. Macroscopic lesions were present in 212 of these and *Salm. gallinarum* was isolated from the faeces of 191.

The Excretion of Salm. gallinarum *in the Faeces of Infected Chickens*

The results of examining faecal swabs taken from 40 chickens at 10 day intervals after infection is shown in Table 6. *Salm. gallinarum* was isolated from the faeces of 35 of these chickens 20 days after infection. The number of faecal-positive chickens then showed a progressive decline until at 60 days after infection only 4 were positive.

TABLE 6

THE RELATIONSHIP BETWEEN DURATION OF INFECTION AND THE EXCRETION OF *Salm. gallinarum* IN THE FAECES

Time after infection (days)	*No. of 40 chickens excreting* Salm. gallinarum *in their faeces*
20	35
30	28
40	12
50	8
60	4

Salm. gallinarum was never present in large numbers in the faeces of carrier chickens. For example, of 19 chickens shown to be excreting *Salm. gallinarum* in their faeces by selenite enrichment only 9 were found to be positive by direct culture of the faeces on to D.C.S.S. medium. About 50 colonies were present on the plate inoculated with faeces from one of these chickens. Only 1 or 2 colonies grew from the faeces of the other 8 chickens. In some cases so few bacteria were excreted that some were shown by selenite medium to be negative at one examination and positive at the next. The administration by mouth of either 0.4 ml. of colocynth or 0.2 ml. of croton oil to 30 infected chickens, 19 of which were excreting *Salm. gallinarum* in their faeces and 11 of which had recently been excreting this organism, had no effect in either increasing the number of excreters or in increasing the numbers of the bacteria excreted by those still shown to be faecal-positive.

The Site of Infection in Faecal Excreters

The results of culturing organs of 30 chickens that were excreting *Salm. gallinarum* in their faeces and a similar number of infected chickens that were not is shown in Table 7. All 60 chickens had been infected approximately 40 days previously. The most significant difference between the 2 groups of chickens was that *Salm. gallinarum* was recovered from the intestinal wall of nearly all the faecal excreters and from only 4 of those that had not been demonstrated to be excreting *Salm. gallinarum* in their faeces; the bile was not commonly infected. The heart, spleen, lung and bone marrow of these 60 chickens were not examined since it was considered that the presence or absence of *Salm. gallinarum* in these organs would not have any bearing on the faecal excretion of this organism.

TABLE 7
THE SITES IN WHICH *Salm. gallinarum* WAS FOUND IN CHICKENS THAT WERE OR WERE NOT FAECAL EXCRETERS

	No. of 30 chickens in which Salm. gallinarum *was found in the*				
Classification	*Intestinal wall*	*Liver*	*Bile*	*Pancreas*	*Kidney*
Faeces +	26	17	6	7	2
Faeces —	4	11	0	10	2

The Presence of Salm. gallinarum *in the Faeces of Chickens after Oral Administration and the Production of Clinical Infection*

Seventy chickens that had been fasted for 18 hours were given approximately 1 million viable *Salm. gallinarum* bacteria in different food materials. The faeces excreted by each chicken during the period of 4 to 6 hours after administration were examined for *Salm. gallinarum* and the results compared with the actual infection rate assessed 3 weeks later. *Salm. gallinarum* was isolated from the faeces of 28 chickens but only 17 of these became clinically infected. Of the 42 chickens from the faeces of which it was not isolated, 14 became clinically infected.

The Time Taken for Demonstrable Blood Agglutinins to Develop

Small amounts of *Salm. gallinarum* culture were given to 144 9 week-old chickens by mouth. Commencing 7 days later, these chickens were submitted to the rapid whole blood agglutination test at intervals of 3 days for 21 days. Only 54 chickens became infected, 10 of which died before agglutinins were demonstrated in their blood. Completely negative results were obtained 7 days after administration of culture. The total number of chickens that were

agglutination-positive at 10 days was 24, at 14 days was 41, at 17 days was 43 and at 20 days was 44. Further examinations did not reveal any more chickens to be positive. All the 44 chickens either died or were found to possess the characteristic lesions of infection when they were submitted to post-mortem examination. No lesions were found in the 90 chickens that were negative to the agglutination test neither were any of them found to be excreting *Salm. gallinarum* in their faeces.

Quite different results were obtained with chickens that were 3 days old when they were infected. Agglutinins were not demonstrated in the blood of the survivors until they were at least 4 weeks old.

The Immunity Following Clinical Infection

The immunity of 20 chickens that had recovered following infection 9 weeks previously, and were not excreting *Salm. gallinarum* in their faeces although they were still positive to the agglutination test, was challenged by giving them 500 million *Salm. gallinarum* bacteria in alkali by mouth. They remained quite well but 9 of 12 control chickens of the same age died. These 20 chickens were then given 100 million *Salm. gallinarum* bacteria intravenously. Although some of them appeared unwell for a short period they rapidly recovered. None of them became faecal excreters as a result of this treatment. A further 40 clinically-recovered chickens were challenged by giving them 50 million *Salm. gallinarum* bacteria by mouth. None of these chickens showed any sign of ill-health or became faecal excreters of *Salm. gallinarum.*

The drinking water of 11 chickens that had been infected 10 to 17 weeks previously was replaced for 1 week by an 18 hour broth culture of *Salm. gallinarum* diluted 1 in 20 with water. Like those used in the previous experiments these chickens had completely recovered from their previous infection; they were not excreting *Salm. gallinarum* in their faeces but were agglutination-positive. During the period they were given infected drinking water, large numbers of *Salm. gallinarum* were found in the faeces of these 11 chickens. At 2 days and at subsequent times after the infected drinking water was replaced by fresh water, *Salm. gallinarum* was not found in the faeces of any of the chickens. They also did not show any signs of ill-health.

DISCUSSION

Although the present study was made more difficult by the fact that chickens do not possess discrete lymph nodes, it is clear that the pathogenesis of *Salm. gallinarum* infection in chickens resembles that of *Salm. typhimurium* infection in mice (Ørskov, Jensen and Kobayashi, 1928). The probable sequence of events is as follows. Following ingestion, some *Salm. gallinarum* bacteria enter the tissues

of the intestine. The remainder are either destroyed or are eliminated in the faeces. A very transient bacteraemia results, the bacteria then becoming localized in the reticulo-endothelial system, particularly in the liver and spleen, although some, undoubtedly, remain in the intestinal wall, probably in the lymphoid follicles. Should the bacteria survive the defence-mechanisms they multiply and a secondary bacteraemia results. This usually commences 3 or more days after infection and lasts, if death does not occur, for up to 7 days. The re-appearance of *Salm. gallinarum* in the faeces and the elevation of the body temperature usually coincides with the onset of the bacteraemia. Following the bacteraemic phase, the bacteria localize in different organs, the one most commonly involved being the heart. It is impossible to say whether the lesions in the intestinal wall, the liver and the spleen are the result of bacterial invasion before, or occur during the secondary bacteraemic phase. Despite the common development and severity of the heart lesions they did not appear to greatly influence the outcome of the chronic phase of the disease. A fatal termination was much more closely correlated with the presence of lesions in the intestinal tract. The survivors usually continued to excrete *Salm. gallinarum* in their faeces for periods of 4 weeks. The proportion of faecal excreters then declined rapidly but since bacilli were isolated from chickens 83 days after infection it would be wise to regard any chickens recovered from infection as potential faecal excreters and a danger to susceptible chickens.

It is noteworthy that no evidence was obtained to support the view that *Salm. gallinarum* might inhabit, and multiply in, the contents of the alimentary tract. In fact all the evidence indicated that any *Salm. gallinarum* bacteria found in the intestinal contents originated from infected foci in the tissues. For example, *Salm. gallinarum* was never isolated from the intestinal contents unless it was also found in the intestinal wall or in one or more organs which communicated with it. Also, when *Salm. gallinarum* bacteria were administered to susceptible chickens by mouth they rapidly disappeared from the faeces and only reappeared when the infection had become generalized. Again, the administration of large numbers of *Salm. gallinarum* bacteria to immune chickens for periods of a week did not result in their colonization in the intestinal contents. The number of bacteria found in the faeces of carriers, too, was always small indicating that if any multiplication occurred in the contents it was very slight. The results indicate that infection in the intestinal wall was the most common source of origin of *Salm. gallinarum* found in faeces. The bile was not commonly found to be infected and, in this respect, the disease differs from many of the salmonella infections of man and other animals. The urinary tract, too, was not commonly involved.

Macroscopically, the heart lesions found in chickens suffering rom the chronic form of the disease closely resembled those described

by Gauger (1937), Biester and Schwarte (1948) and Hall, *et. al* (1949) as occurring in the chronic form of the natural disease. These authors, however, referred to their lesions as necrotic foci but the lesions found in the present study were definitely proliferative and cellular in nature and no obvious necrosis of the muscle fibres of the heart was ever noted.

The observation that 1-day-old chicks were the most susceptible to fatal infection and that resistance increased with age, as far as immature chickens were concerned, was not unexpected. Neither, at first, was the fact that mature female chickens were more susceptible than immature females since it was felt that this might be associated with the increased strain of egg production. However, since mature cockerels also possessed this increased susceptibility the reason for it remains unknown.

A knowledge of their expected food consumption is a great advantage when flocks of chickens are to be treated for any disease by means of chemotherapeutic agents since it is desirable, and often essential, that these agents should be given either in the food or the drinking water. Since the food consumption of chickens suffering from experimental fowl typhoid was approximately half that of healthy chickens it would be wise during treatment to increase the concentration of chemotherapeutic agents added to food to at least twice that estimated as a result of pharmacological studies carried out on healthy chickens.

CONCLUSIONS

A disease closely resembling fowl typhoid was consistently produced in chickens by the oral administration of *Salm. gallinarum*.

Infection took place *via* the intestinal tract. Localization then occurred, principally in the intestinal wall, the liver and the spleen. This was followed by a bacteraemic phase which terminated either in death or in the chronic form of the disease which was characterized by the development of proliferative lesions, mainly in the heart wall. Fatal cases of the chronic form, however, were usually associated with the presence of intestinal lesions.

One-day-old chicks were the most susceptible to fatal infection. Susceptibility then decreased with age, but increased again with maturity. Both sexes were equally susceptible.

Of 300 infected 9 week-old cockerels, 45 per cent. died in the acute phase of the disease and 15·7 per cent. in the chronic. Deaths usually commenced to occur 7 days after infection reaching a peak on the 8th to 14th day; very few deaths took place after the 20th day.

Some chickens continued to excrete *Salm. gallinarum* in their faeces for 2 to 3 months after infection, but the number of bacteria excreted was invariably small. The presence of *Salm. gallinarum* in the faeces was always associated with foci of infection in the tissues,

particularly in the intestinal wall. The gall bladder was much less commonly involved.

The rapid whole blood agglutination test was very reliable in identifying infected chickens when it was employed 20 or more days after infection.

When clinically-recovered chickens were exposed to a massive infection by mouth or intravenously they neither became ill nor became faecal excreters of *Salm. gallinarum.*

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Mr. W. E. Crabb, A.I.M.L.T., and Miss G. Anderson for their capable technical help. I am also grateful to Dr. R. F. Gordon for providing the facilities for carrying out this work and to Dr. C. le Q. Darcel for help in interpreting histological findings. The photographs were taken by Mr. W. E. Crabb.

REFERENCES

Biester, H. E., and Schwarte, L. H. (1948). *Diseases of Poultry.* 2nd Edition, The Iowa State College Press, Ames, Iowa.

Gauger, H. C. (1937). *Bull. agric. expt. Sta. N. Carolina State. Coll. Agric.,* No. 53. 1.

Ørskov, J., Jensen, K. A., and Kobayashi, K. (1928). *Z. ImmunForsch.,* **55**, 34.

Gordeuk, S., Glantz, P. J., Callenbach, E. W., and Thorp, W. T. S. (1949). *Poult. Sci.,* **28**, 385.

Gordon, R. F., and Brander, G. C. (1942). *Vet. Rec.,* **54**, 275.

Hall, W. J., Legenhausen, D. H., and MacDonald, A. D. (1949). *Poult. Sci.,* **28**, 344.

Hobbs, B. C., and Allison, V. D. (1945), *Mon. Bull. Min. Hlth. Publ. Hlth Lab. Serv.,* **4**, 63.

Miles, A. A., and Misra, S. S. (1938). *J. Hyg. Camb.,* **38**, 732.

Smith H. Williams, (1954a). *Brit. J. exp. Path.,* **35,** 447; (1954b) *Nature,* **174**, 45; (1955). *J. comp. Path.,* **65,** 55

Smith, H. Williams, and Buxton, A. (1951). *Brit. med. J.,* i, 1478.

Wilson, J. E. (1946). *Vet. Rec.,* **58**, 269.

[*Received for publication, June 10th,* 1954]

LEGENDS TO ILLUSTRATIONS

Plate 1. Normal heart and heart of 12 week-old chicken 20 days after infection showing white nodular lesions in myocardium. Approx. natural size.

Plate 2. Duodenum of 12 week-old chicken 20 days after infection. The distal limb has been opened to show the white proliferative lesions. One lesion is also seen in the middle of the pancreas. Approx. natural size.

To face page 54

J. Comp. Path. 1955. Vol. 65.

THE CHEMOTHERAPY OF EXPERIMENTAL FOWL TYPHOID IN FOWLS (*GALLUS DOMESTICUS*)

By

H. Williams Smith

Animal Health Trust, Houghton Grange, Huntingdon

INTRODUCTION

The sulphonamides, particularly sulphamerazine and sulphaquinoxaline, have been found to reduce the mortality rate in most laboratory and field studies on fowl typhoid (Hammond, 1945; Moore, 1946; Holtman and Fisher, 1947; Hall, MacDonald and Legenhausen, 1949; Alberts, 1950; Boney, 1951; Cox and Craig, 1952). Very little information is available as to whether chickens so treated remain carriers of infection. Other chemotherapeutic agents have been investigated to a much less extent. Prier and Alberts (1951) found two intraperitoneal injections of aureomycin to have little effect upon the course of the experimental disease in chicks. Recently, Grumbles, Wills and Boney (1954) using turkeys and Smith (1954b) using chickens found furazolidone to be of considerable value in the treatment of the experimental form of the disease. Grumbles, *et al.* reported that recovered turkeys may become carriers of infection. Smith, however, employing a higher dose level found carriers to be rare.

The purpose of this paper is to report on the value of sulphamerazine, sulphaquinoxaline, sulphamezathine, nitrofurazone, furazolidone, penicillin, dihydrostreptomycin, chloramphenicol, aureomycin and terramycin in the treatment of the experimental disease, the characteristics of which have been described previously (Smith, 1955). These agents were usually first studied in-vitro and on infected one-day-old chicks. They were then tested more critically on infected 9-week-old chickens. The choice of route of administration and dosage in these chickens was decided after considering the results of the in-vitro tests and those of observations on blood levels of these agents obtainable in chickens, made recently (Smith, 1954a) or during the course of the present work; the aim was to maintain, where possible, adequate bacteriostatic blood levels throughout the whole period of treatment. The development of agglutinins and the faecal excretion of *Salm. gallinarum* by treated chickens was also studied as well as the treatment of chronic carriers. Since furazolidone was found to be superior to all the other agents, it was examined further to find a method of administration applicable to the treatment of natural outbreaks of the disease, particular attention being paid to its effect on the mortality and carrier rates, the development of agglutinins and resistance to re-infection.

MATERIALS AND METHODS

Chickens. Sex-linked Brown Leghorn x Barred Rock cockerels from a Salmonella-free flock were used when they were either one-day-old

(approx. weight 40g.) or 9-weeks-old (approx. weight 0.75 kg.). The former are referred to throughout as chicks and the latter as chickens. Both were kept on wire-mesh floors while under experiment. The chickens were maintained on a commercial mash, the composition of which has been described previously (Smith, 1955). A somewhat similar mash was fed to the chicks, the main difference being that half of the fish meal was replaced by skimmed milk powder.

Salm. gallinarum *cultures.* Seven strains of *Salm. gallinarum* isolated from different outbreaks of fowl typhoid were used for the in-vitro sensitivity tests.

Chemotherapeutic agents. Crystalline sodium penicillin G of a potency of 1617 units/mg. was employed. Dihydrostreptomycin was used as the sulphate. The hydrochlorides of both aureomycin and terramycin were employed, buffered with sodium glycinate for parental administration. Sulphamerazine, sulphaquinoxaline, sulphamezathine, nitrofurazone, furazolidone and chloramphenicol were used in powder form. They were administered in the mash to the chicks and in capsules to the chickens. Sodium salts of the sulphonamides were prepared for in-vitro tests.

In-vitro sensitivity tests. A suitable range of two-fold serial dilutions of the chemotherapeutic agents was made in 5 ml. of liquid medium. Each series of tubes and a control tube containing medium but no agent was then inoculated with a suitably-diluted *Salm. gallinarum* culture. The tubes were incubated for 24 hours at 37°C. The lowest concentration of the agent that completely inhibited bacterial multiplication, as judged by visual examination, was recorded as the minimal inhibitory concentration. The medium used for the sulphonamide tests was nutrient broth from which sulphonamide-inhibitors had been removed by means of lysed horse erythrocytes according to the method of Evans (1948). The bacterial inoculum was approximately 100 bacteria. For tests with the other agents ordinary nutrient broth at pH 7.2 and an inoculum of approximately 100,000 bacteria was used.

Method of producing infection. Chickens were infected by giving them 50 million viable *Salm. gallinarum* bacteria of one strain, strain 9, in alkaline medium by mouth (Smith, 1955). This dose was always sufficient to cause a 100 per cent. infection rate. A similar technique was employed in the case of chicks except that they were not starved before administration and were given 0.03 g. instead of 0.3 g. of alkali.

The evaluation of the chemotherapeutic agents in vivo. Treatment of chicks, in groups of 30, was always commenced 24 hours after infection and continued for 10 days when the chicks were moved to fresh accommodation. Observations were carried out for 30 days, the survival times of all chicks being recorded. A control group of 30 chicks was always included whenever any agent was being evaluated. No significant difference was noted between the course of the disease in different groups of control chicks and, consequently, the results for only one group are quoted.

The 9-week-old chickens were also employed in groups of 30 and, initially, treatment was commenced 4 days after infection and continued for 6 days. At 21 days after infection the faeces of all surviving chickens were examined for the presence of *Salm. gallinarum* and the blood for agglutinins to this organism. Those that were shown to be excreting *Salm. gallinarum* in their faeces were immediately re-treated for 6 days and their faeces re-examined 5 days later. In view of the satisfactory results obtained

with furazolidone various methods of treatment were studied with this agent. The resistance of some of the groups of chickens treated with furazolidone to re-infection was examined by giving them a similar dose of *Salm. gallinarum* as had been used to produce the initial infection. The survival times of such chickens were noted and their blood and faeces examined 21 days after re-infection.

Controls consisting of groups of 30 untreated chickens were always included in each experiment but for the same reasons given in the case of the chicks, the results for each group are not quoted.

The method of examining faeces for *Salm. gallinarum* and blood for agglutinins to this organism had been described previously (Smith, 1955). The method of examining the organs of chickens for *Salm. gallinarum* was also the same except that, in the present study, the whole of the heart and spleen, and approximately one-third of the liver of each chicken were pooled before they were cultured.

RESULTS

The in-vitro Sensitivity of Salm. gallinarum *to Chemotherapeutic Agents*

The sensitivity of 7 strains of *Salm. gallinarum* to furazolidone, sulphamerazine, sulphaquinoxaline, sulphamezathine, dihydrostreptomycin, chloramphenicol, aureomycin, terramycin and penicillin is shown in Table I. Furazolidone was the most active agent tested, 0.5 μg./ml. being sufficient to prevent the growth of 6 of the 7 strains. Tests showed that this concentration was usually bactericidal and not merely bacteriostatic as was the case with the

TABLE I.

THE SENSITIVITY OF SEVEN STRAINS OF *Salm. gallinarum* TO CHEMOTHERAPEUTIC AGENTS.

Agent	*Minimal inhibitory concentration (ug/ml.) for strain number*						
	1	2	3	4	5	6	9
Furazolidone	0.5	0.5	0.5	0.5	1.0	0.5	0.5
Sulphamerazine	40	80	80	80	80	80	40
Sulphaquinoxaline	320	160	320	320	160	80	80
Sulphamezathine	320	320	320	640	320	320	160
Dihydrostreptomycin	10	10	10	10	5	10	10
Chloramphenicol	10	5	5	10	10	10	10
Aureomycin	10	10	10	10	10	10	5
Terramycin	10	10	10	10	10	10	10
Penicillin	32	16	16	32	32	16	16

other agents in their minimal inhibitory concentrations. Sulphamerazine was the most active of the three sulphonamides tested, 40 to 80 μg./ml. preventing bacterial growth compared with 80 to 640 μg./ml. of sulphamezathine and sulphaquinoxaline. Dihydrostreptomycin, chloramphenicol, aureomycin and terramycin were equally active, their minimal inhibitory concentrations being 5 to 10 μg./ml. Concentrations as high as 16 to 32 μg./ml. of penicillin was required to inhibit bacterial growth.

The Addition of Furazolidone to the Diet of Healthy Chickens

When mash containing 0.02 and 0.04 per cent. furazolidone was fed continuously to groups of 39 one-day-old chicks and to groups of 53 four-week-old chickens for 3 weeks, none of them died and no obvious signs of ill-health were noted. Growth was not so rapid as in control chickens, however. The total increase in weight of the day-old chicks given 0.02 per cent. furazolidone was 12.4 per cent. less than the controls and those given 0.04 per cent., 17.2 per cent. less. Two weeks after administration was discontinued the corresponding figures were only 1.6 and 3.1 per cent. respectively. Similarly, the total increase in weight of the 0.02 per cent. group and the 0.04 per cent. group of four-week-old chickens was 3.8 per cent. and 15.3 per cent. respectively less than the controls after 3 weeks. The figures had decreased to 1.7 and 5.1 per cent., respectively, 2 weeks after administration had ceased indicating, as in the case of the day-old chicks, that the depression in growth had been made good.

The Treatment of Infected Chicks

The effect of 10 days treatment with different agents on the course of the disease in groups of 30 infected chicks is illustrated in Table 2. All 30 untreated chicks died within 5 days of infection. Furazolidone, at a concentration of 0.02 per cent. in the food, was by far the most effective agent, 28 of 30 chicks surviving, only one of which was excreting *Salm. gallinarum* in its faeces at the end of the experiment. Terramycin (50 mg./kg.) given intramuscularly once daily, and chloramphenicol, as 0.5 per cent. in the mash, were very effective in controlling the disease during the period the chicks were under treatment; only 2 died in each group and the remainder appeared quite healthy. When treatment was discontinued the chicks soon became unwell again and the mortality rate rose rapidly; at the end of the experiment the number of survivors in each group was only 6 and 3 respectively. The feeding of mash containing 0.5 per cent. sulphamerazine or sulphamezathine or 0.2 per cent. sulphaquinoxaline reduced the mortality rate, deaths ceasing to occur during the treatment period after the 7th day in the sulphamerazine and sulphaquinoxaline groups. However, as with the chicks treated with terramycin and chloramphenicol, the mortality rate increased

TABLE 2.

THE FREQUENCY DISTRIBUTION OF THE SURVIVAL TIMES OF GROUPS OF 30 INFECTED CHICKS TREATED WITH DIFFERENT AGENTS.

Agent	*Days after infection*																*Survivors*	
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18+	*No.*	%
Furazolidone (0.02% in food)	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	28	92.5
Terramycin (50 mg./kg. once daily)	0	2	0	0	0	0	0	0	0	0	1	5	5	3	2	6	6	19.8
Nitrofurazone (0.02% in food)	5	12	5	1	1	0	0	0	0	0	1	0	0	0	0	0	5	16.5
Dihydrostreptomycin (50 mg./kg. once daily)	0	1	3	10	2	1	3	3	1	0	0	1	1	0	0	0	4	13.2
Chloramphenicol (0.5% in food)	1	1	0	0	0	0	0	0	0	1	3	9	9	3	0	0	3	9.9
Sulphamerazine (0.5% in food)	1	1	2	4	1	0	0	0	0	0	3	2	5	3	2	3	3	9.9
Sulphaquinoxaline (0.2% in food)	1	2	3	5	2	0	0	0	0	1	5	5	2	0	1	1	2	6.6
Sulphamezathine (0.5% in food)	0	3	3	3	1	1	2	2	1	2	0	2	3	1	2	2	2	6.6
Penicillin (150 mg./kg. once daily)	1	6	7	7	8	1	0	0	0	0	0	0	0	0	0	0	0	0
None	6	17	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Treatment was applied for 10 days commencing 1 day after infection.
Dihydrostreptomycin, terramycin and penicillin were adminstered by intra-muscular injection.

when treatment ceased, the final number of survivors in the sulphamerazine, sulphaquinoxaline and sulphamezathine groups being 3, 2 and 2 respectively. Dihydrostreptomycin (50 mg./kg.) given intramuscularly once daily, had a less marked effect on the mortality rate than the agents previously mentioned, the number of survivors being 4. Penicillin (150 mg./kg.) given intramuscularly once daily, and nitrofurazone, 0.02 per cent. in the food, had only a very slight effect on the course of the disease.

Aureomycin therapy was not studied because of the difficulty of giving repeated intravenous injections to young chicks; injection by other routes was not attempted owing to the irritant nature of this agent. Oral aureomycin, as well as terramycin, therapy was not considered worthy of investigation since these agents are very poorly absorbed from the alimentary tract of chickens. (Smith, 1954a)

The Feeding of Low Concentrations of Furazolidone to Chicks before Infection

None of 20 chicks that had been maintained on mash containing 0.004 per cent. furazolidone since hatching died after being given an infective dose of *Salm. gallinarum* when they were 4 days old. Seven of them were still excreting *Salm. gallinarum* in their faeces 3 weeks later although they had been given this diet continuously until 2 days before testing. When the concentration was reduced further to 0.002 per cent., 5 of 20 chicks died after infection and another 5 became carriers. Eighteen of 20 control chicks fed on ordinary mash died; the remaining 2 became faecal excreters.

The Treatment of Infected Chickens

The survival times of groups of 30 nine-week-old chickens treated with furazolidone (25 mg./kg. once daily by mouth), sulphamerazine (300 mg./kg. followed by 150 mg./kg. twice daily by mouth), dihydrostreptomycin (25 mg./kg. twice daily intramuscularly), chloramphenicol (150 mg./kg. twice daily by mouth), terramycin (50 mg./kg. once daily intramuscularly), aureomycin (50 mg./kg. once daily intravenously) and penicillin (150 mg./kg. once daily intramuscularly) are illustrated in Table 3. Treatment was applied for 6 days commencing 4 days after infection. Furazolidone, sulphamerazine, chloramphenicol and terramycin had a very beneficial effect on the course of the disease, the number of chickens surviving in each group being 29, 27, 26 and 28 respectively compared with only 10 in the untreated group. Aureomycin and dihydrostreptomycin were less effective, the number of survivors being 24 and 20 respectively. Penicillin treatment was of no value.

The two chloramphenicol-treated chickens that died 18 and 19 days after infection possessed lesions characteristic of the acute form of the disease, lesions normally found only before 14 days of infection in untreated chickens (Smith, 1955). Of another 10 chickens in which chloramphenicol therapy was applied for 6 days commencing

TABLE 3.

THE FREQUENCY DISTRIBUTION OF THE SURVIVAL TIMES OF GROUPS OF 30 INFECTED CHICKENS TREATED WITH DIFFERENT AGENTS.

Agent	*Days after infection*														*Survivors*	
	7	8	9	10	11	12	13	14	15	16	17	18	19	20+	*No.*	%
Furazolidone (25 mg./kg. once daily)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	29	96.7
Terramycin (50 mg./kg. once daily)	0	1	0	0	0	1	0	0	0	0	0	0	0	0	28	93.3
*Sulphamerazine (150 mg./kg. twice daily)	0	0	0	0	0	0	0	0	0	0	0	0	1	2	27	90.0
Chloramphenicol (150 mg.kg. twice daily)	0	0	0	0	0	0	0	0	0	0	0	1	1	2	26	86.7
Aureomycin (50 mg./kg. once daily)	0	0	0	0	0	1	0	3	1	0	0	0	1	0	24	80.0
Dihydrostreptomycin (25 mg./kg. twice daily)	0	0	0	0	0	0	0	1	0	3	2	1	1	2	20	66.6
Penicillin (150 mg./kg. once daily)	0	2	3	4	3	1	0	1	0	1	1	0	2	0	12	40.0
None	0	2	3	4	4	1	1	3	0	1	0	1	0	0	10	33.3

Treatment was applied for 6 days commencing 4 days after infection. Terramycin and penicillin were administered intramuscularly, aureomycin intravenously and the other agents by mouth in capsules.

*The first dose of sulphamerazine was 300 mg./kg.

2 days, instead of 4 days, after infection, 3 chickens died of the acute form of the disease 16, 17 and 19 days after infection. Such fatal relapses were not noted in chickens treated with other agents, all chickens dying 14 or more days after infection possessing lesions of the chronic type.

The Presence of Agglutinins in the Blood and Salm. gallinarum *in the Faeces of Treated Chickens: the Effect of Re-treatment*

The results of agglutination tests and faecal culture of the surviving chickens of the previous experiment performed 21 days after infection are shown in Table 4. The results are expressed as percentages in order that the effects of the different agents can be more easily compared. The figures quoted for untreated chickens are compiled from the survivors of 2 control groups. Agglutinins to *Salm. gallinarum* were not demonstrable in the blood of any of the chickens that had been treated with furazolidone; neither were they excreting this organism in their faeces. In contrast, all surviving chickens after sulphamerazine, dihydrostreptomycin, terramycin, penicillin and aureomycin treatment, as well as the untreated controls, were agglutination-positive and most of them (70.8 to 88.9 per cent,) were excreting *Salm. gallinarum* in their faeces. Only 80.8 per cent. of the chloramphenicol-treated survivors were positive to the agglutination test although 92·3 per cent. were excreting *Salm. gallinarum* in their faeces; all were agglutination-positive when re-tested 10 days later. This delay in the development of agglutinins was a fairly common feature of chloramphenicol treatment.

TABLE 4.

THE PRESENCE OF AGGLUTININS IN THE BLOOD AND *Salm. gallinarum* IN THE FAECES OF CHICKENS AFTER TREATMENT: THE EFFECT OF RE-TREATMENT.

Agent	*Treated chickens* *		*Re-treated chickens*
	Agglutination+ %	*Faeces+* %	*Faeces+* %
Furazolidone	0	0	—
Terramycin	100	78.5	28.5
Sulphamerazine	100	88.9	53·4
Chloramphenicol	80.8	92.3	20.0
Aureomycin	100	70.8	—
Dihydrostreptomycin	100	84.2	69.2
Penicillin	100	100	—
Untreated	100	82.6	66.6

*Examinations performed 21 days after infection.

The results of re-treating the chickens that were excreting *Salm. gallinarum* in their faeces with a similar course to that previously given is also shown in Table 4. The survivors of the penicillin-treated group were not re-treated in view of the lack of response to this agent. Neither were those of the aureomycin-treated group since a considerable amount of sloughing had occurred at the sites of intravenous injection in some of the chickens as a result of the first course of treatment. Re-treatment with sulphamerazine and dihydrostreptomycin had no effect in decreasing the excreter-rate, 69.2 per cent. and 53.4 per cent. respectively of the chickens in each group still continuing to excrete *Salm. gallinarum* in their faeces after re-treatment compared with 66.6 per cent. in the untreated group .The number of excreters decreased in the chloramphenicol and terramycin-treated groups, the figures after re-treatment being 20 per cent. and 28.5 per cent. respectively.

The Presence of Lesions in Chickens after Treatment

The chickens used in the previous experiments were killed approximately 5 weeks after infection and classified according to the presence and degree of severity of the macroscopic lesions associated with the chronic disease (Smith, 1955). The results are illustrated in Table 5. There was very little difference between the chickens treated with sulphamerazine, terramycin and penicillin, and the untreated chickens. Lesions were found less commonly in those treated with dihydrostreptomycin and chloramphenicol while no lesions were found in any one of the 29 survivors that had been treated with furazolidone. *Salm. gallinarum* was not isolated from the organs of any of these 29 chickens.

TABLE 5.

THE PRESENCE OF MACROSCOPIC LESIONS IN CHICKENS AFTER TREATMENT WITH DIFFERENT AGENTS.

Agent	*Severe lesions* %	*Mild lesions* %	*No lesions* %
Furazolidone	0	0	100.0 *
Terramycin	46.1	38.4	15.5
Sulphamerazine	44.0	24.0	32.0
Chloramphenicol	31.8	18.2	50.0
Dihydrostreptomycin	36.8	15.8	47.4
Penicillin	66.6	0	33.3
Untreated	55.6	11.1	33.3

Examinations were made 5 weeks after infection.
*The organs were bacteriologically negative also.

The Effect of Treatment with Furazolidone in the Food

The effect of different methods of administering furazolidone on the mortality rate of groups of 30 infected chickens is illustrated in Table 6. The figures quoted refer solely to the deaths that occurred after the commencement of treatment. The results of agglutination tests and faecal culture on the survivors is also shown. The feeding of mash containing 0.02 per cent. furazolidone was quite satisfactory in controlling the disease when it was given for 6 days commencing 2 to 6 days after infection; the mortality rate never exceeded 6.6 per cent. neither did the carrier rate amongst the survivors exceed 5.3 per cent. A higher mortality rate, 10.0 per cent., and a higher carrier rate, 14.8 per cent., resulted when the period of treatment was reduced to 3 days. Increases in these figures were also noted, 20.0 per cent. and 33.3 per cent. respectively, when the concentration was reduced to 0.01 per cent. The mortality rate and faecal excreter rate increased when treatment was not applied until 8 to 10 days after infection. This was not unexpected as some of the chickens

Table 6.

The treatment of Groups of 30 Infected Chickens with Furazolidone in the Food.

Percentage in food	*Treatment*		*Died*	*Survived*	
	Commencement after infection (days)	*Duration (days)*	%	*Agglutination* + %	*Faeces* + %
0.01	4	6	20.0	33.3	33.4
0.02	2	6	3.3	3.4	3.4
0.02	4	3	10.0	14.8	14.8
0.02	4	6	3.0	0	0
0.02	4	12	3.3	0	0
0.02	6	6	6.6	21.4	5.3
0.02	8	6	10.0	44.4	11.1
0.02	10	6	26.7	62.5	31.3
0.02*	10	6	10.0	55.5	25.9
0.02	10	10	20.0	66.6	4.2
0.04	10	10	13.3	64.3	0
0.04	10	10	10.0	74.0	0
	Untreated		70	100	90

*Capsule containing 25 mg./kg. furazolidone given by mouth at commencement of treatment.

were so ill that they ate very little. It is noteworthy in this respect that the mortality rate was 26.7 per cent. amongst chickens in which treatment was commenced 10 days after infection and maintained for 6 days compared with 10 per cent. in a similar group given also one capsule of furazolidone, (25 mg./kg) by mouth at the commencement of treatment. The faecal excreter rate was also high in both these groups, 31.3 and 25.9 per cent. respectively. In another group in which treatment was continued for 10 days instead of the customary 6, however, the excreter rate was only 4.2 per cent. Very satisfactory results were obtained in 2 groups of chickens, both of which were given 0.04 per cent. instead of 0.02 per cent. furazolidone in the food for 10 days commencing 10 days after infection; the mortality rate was 13.3 per cent. and 10.0 per cent. and none of the 60 chickens were found to be excreting *Salm. gallinarum* in their faeces after treatment.

When treatment was commenced 2 or 4 days after infection only those chickens excreting *Salm. gallinarum* in their faeces after treatment developed agglutinins to that organism. When the time of commencing treatment was delayed to 6, 8 or 10 days after infection many more of the surviving chickens developed agglutinins, the greater the delay in commencing treatment the greater the number of chickens that were agglutination-positive. The number that were agglutination-positive was no longer related to the faecal excretion rate. For example, in the 2 groups given 0.04 per cent. furazolidone for 10 days commencing 10 days after infection, the percentage of chickens that were positive to the agglutination test was 64.3 and 74.0, yet none of them were excreting *Salm. gallinarum* in their faeces.

The Disappearance of Agglutinins to Salm. gallinarum *from the Blood of Chickens treated with Furazolidone*

Of 69 infected chickens in which treatment with 0.04 per cent. furazolidone in the food was commenced 10 to 12 days after infection and continued for 10 days, 49 were positive to the agglutination test when examined 3 weeks after infection. Not one of the 69 chickens was excreting *Salm. gallinarum* in its faeces. The number that were agglutination-positive at 4, 5, 6 and 7 weeks after infection was 40, 18, 14 and 6 respectively. The chickens were then killed. No lesions were noted in any one of them and *Salm. gallinarum* was only isolated from the organs of one of them; this chicken was agglutination-positive.

The Immunity of Chickens whose Initial Infection had been Cured by Furazolidone Therapy

The results of attempting to re-infect groups of chickens which had been cured of their initial infection with *Salm. gallinarum* by different regimens of furazolidone therapy is shown in Table 7. A similar dose of culture as that normally used to produce infection

was given by mouth approximately 4 weeks after the chickens had been exposed to their initial infection. Chickens in which treatment of the initial infection had been commenced either 2 or 4 days after exposure to infection possessed very little resistance to re-infection, 53·3 and 43·3 per cent. respectively dying. A greater degree of resistance to re-infection was noted in the groups in which treatment of the initial infection had been commenced later. When the delay had been 10 days, none of 61 chickens died when re-infection was attempted and only a small proportion commenced to excrete *Salm. gallinarum* in their faeces. All chickens were agglutination-positive after re-infection.

TABLE 7.

THE IMMUNITY OF CHICKENS WHOSE INITIAL INFECTION HAD BEEN CURED BY FURAZOLIDONE THERAPY.

No. of chickens cured of initial infection in group	*Details of treatment of initial infection*			*Cured chickens that died when re-infected* %	*Survivors faecal +* %
	Drug in food %	*Commencement after infection (days)*	*Duration (days)*		
30	0.02	2	6	53·3	71·4
30	0.02	4	6	43·3	58·8
53	0.02	6	6	20.7	45·7
52	0.02	8	6	9.6	23·4
33	0.02	10	6	0	21.2
28	0.04	10	10	0	10.7

The Treatment of Faecal Carriers with Furazolidone

The effect of furazolidone treatment on chickens that had been infected 4 to 5 weeks previously but were all excreting *Salm. gallinarum* in their faeces is shown in Table 8. Furazolidone, 0.02 per cent. for 6 days, had a definite effect in decreasing the carrier rate, only 20.8 per cent. of chickens continuing to excrete *Salm. gallinarum* in their faeces compared with 61.6 per cent. of the untreated group. A more marked effect was observed with 0.04 per cent. furazolidone, the percentage of chickens excreting *Salm. gallinarum* after treatment for 8 to 10 days being only 6.6 and 10.8 respectively. The organs of the 37 chickens that had been treated with 0.04 per cent. furazolidone for 10 days were also cultured; 5 of these chickens (13.7 per cent.) were found to be infected. Very severe lesions were present and it was not surprising that furazolidone treatment did not sterilize them of bacterial infection.

Table 8.

The Treatment of Faecal Carriers with Furazolidone.

*Number in group**	*Treatment*		*Chickens still faecal +*	
	Drug in food %	*Duration (days)*	No.	%
24	0.02	6	5	20.8
30	0.04	8	2	6.6
37	0.04	10	4	10.8
54	None		33	61.1

*These chickens had been infected 4-5 weeks previously.

DISCUSSION

The majority of the chemotherapeutic agents were effective in preventing most of the mortality associated with the experimental disease in the 9-week-old chickens. These findings are in conformity with the in-vitro results bearing in mind that the dosage of these agents was sufficient to maintain a concentration in the blood during the greater part of the treatment period considerably higher than that required to inhibit growth of *Salm. gallinarum* in-vitro. It was only when treated chickens were examined for faecal excretion of *Salm. gallinarum* and for lesions, and when the course of the disease in treated one-day-old chicks was considered, that the great superiority of furazolidone over the other agents became apparent. This superiority is undoubtedly related to the fact that furazolidone is bactericidal in low concentrations while the other agents are only bacteriostatic. Agents like chloramphenicol and terramycin almost completely controlled the disease in chicks during the period of treatment but afterwards deaths rapidly commenced to occur. Since cross-infection was shown to be a rare event under the conditions in which the chicks were maintained, these relapses were undoubtedly due to the fact that the *Salm. gallinarum* bacteria in the tissues were only prevented from multiplying and, when bacteriostatic levels of these agents were no longer present, multiplication commenced and the normal disease process was resumed. Fatal relapses were very much less common in the 9-week-old chickens treated with the bacteriostatic agents, a fact that is probably related to the greater resistance of those chickens to fatal infection compared with chicks and to the fact that they were able to develop some immunity during the period of treatment. Instead, the chronic form of the disease occurred associated with lesions in the internal organs and excretion of *Salm. gallinarum* in the faeces. Immunity was produced more slowly

by chloramphenicol-treated chickens, as shown by the delay in the production of agglutinins and fatal relapses that occurred in some of these chickens. This was probably related to the greater bacteriostatic efficiency of chloramphenicol compared with the other agents and is not uncommonly found during the treatment of typhoid infection in human beings. The almost complete absence of the undesirable sequelae referred to above after furazolidone treatment can be satisfactorily explained on the basis of its bactericidal action. Bactericidal blood levels were found during the first 8 hours of administering a single dose of 25 mg./kg. to chickens although absorption appeared to be erratic, a fact possibly associated with the very low solubility of this agent in water.

It is not always easy to forecast the value of a chemotherapeutic agent in natural outbreaks from studies carried out on the experimental form of a disease. From the present work, however, it can be deduced that agents such as the sulphonamides, chloramphenicol, terramycin, dihydrostreptomycin and aureomycin would be of limited value in the treatment of natural outbreaks of fowl typhoid, particularly when it is appreciated that it would be difficult, and in some cases impossible, under field conditions to institute the methods of treatment that have been studied. For instance, it would not usually be practicable to give chickens 6 intramuscular injections of terramycin or streptomycin let alone a similar number of injections of aureomycin intravenously. Again, from the economic point of view, the cost of many of the agents used would be prohibitive. Under certain conditions, too, the use of these bacteriostatic agents would be dangerous since the large number of carriers that arise following treatment would be a constant source of infection to susceptible chickens and further outbreaks would probably occur. Most workers have shown this to be the case when sulphonamide therapy has been applied in natural outbreaks of fowl typhoid.

The position with regard to furazolidone therapy may well be different provided adequate dosage is adopted and strict sanitary measures implemented. The number of chickens that became faecal excreters was very small when treatment with 0.02 per cent. furazolidone was commenced 4 days after infection. The number increased with the duration of infection before treatment. It would seem logical, therefore, when this agent is employed in natural outbreaks in which chickens in all stages of infection would be found, to administer furazolidone at a concentration of 0.04 per cent. in the mash for a period of 10 days since this course of treatment did not result in any of the chickens, in which administration was commenced as long as 10 days after infection, becoming faecal excreters. The mortality rate, too, might be reduced by giving ailing chickens a capsule containing furazolidone, by mouth at the rate of 25 mg./kg. Since chickens treated in the early stages of the experimental disease were susceptible to re-infection, an infected flock should be moved

to clean premises at the end of the course of treatment and, if possible, a further course given. Such measures might well prevent a further outbreak of infection. It should also be remembered that, apart from those in the early stages of infection, many chickens in an infected flock would themselves be un-infected when treatment was instituted and would be fully susceptible to future infection. Should it be impossible to move chickens to fresh ground, it would probably be worthwhile following the first course of treatment with the continuous feeding of mash containing low concentrations of furazolidone, say 0.004 to 0.01 per cent., since 0.004 per cent. of furazolidone prevented the occurrence of fatal infection in chicks fed on mash containing this concentration before being given an infective dose of *Salm. gallinarum*. Cooper (1954) found that the feeding of mash containing these low concentrations of furazolidone for 6 weeks did not depress the growth rate of chicks.

The development of agglutinins in chickens treated early in the course of the disease with furazolidone was almost invariably indicative of a focus of infection that had not been sterilized. This did not apply in chickens treated later in the course of the disease since many of these became positive to the agglutination test although they were no longer infected; some, in fact, had produced agglutinins even before treatment was commenced. Consequently, a policy of eradication based on the agglutination test applied soon after the completion of treatment in outbreaks of the natural disease might well result in the elimination from the flock of chickens that were, in fact, no longer infected. Most of the chickens treated 10 days after infection that developed agglutinins, but were negative on faecal culture, became agglutination-negative after a comparatively short period of time. It is possible, therefore, that agglutination tests carried out 6 to 8 weeks or more after the conclusion of treatment may be of more significance than those carried out earlier since they would identify, in the main, only chickens possessing an infected focus.

The effectiveness of furazolidone in the treatment of chronic carriers of infection increases the possibility that this and similar agents may have a much wider application than in the treatment of salmonella infections in poultry. These might, for example, be of value in the treatment of such diseases as typhoid in human beings.

CONCLUSIONS

Furazolidone was greatly superior to sulphonamides, dihydrostreptomycin, chloramphenicol, terramycin and aureomycin in the treatment of experimental fowl typhoid in one-day-old chicks and 9-week-old chickens. This superiority was associated with the fact that furazolidone was bactericidal for *Salm. gallinarum* in low concentrations whereas the other agents were only bacteriostatic. Penicillin treatment was valueless.

The occurrence of faecal excreters of *Salm. gallinarum* was very uncommon amongst chickens that had been treated with furazolidone. A concentration of 0.04 per cent. was necessary to achieve this when furazolidone was given in the mash, administration being continued for 10 days. Faecal excreters were found more frequently when the concentration and the period of administration were decreased. Most of the chickens treated with the other agents became faecal excreters.

Chickens treated with furazolidone early, but not late, in the course of the experimental disease developed very little immunity as evidenced by the absence of agglutinins to *Salm. gallinarum* in their blood and their susceptibility to re-infection. The need for instituting strict methods of sanitary control during the treatment of natural outbreaks is therefore indicated.

Furazolidone was also of considerable value in the treatment of chronic carriers of infection.

Apart from a slight depression in the growth rate, the continuous feeding of healthy chicks and young chickens on mash containing 0.02 or 0.04 per cent, furazolidone for 3 weeks was not accompanied by any signs of toxicity.

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Mr. W. E. Crabb, A.I.M.L.T., and Miss G. Anderson for their capable technical help. I am also grateful to Dr. R. F. Gordon for providing the facilities for carrying out this work.

The aureomycin was kindly supplied by Lederle Laboratories, the chloramphenicol by Allen and Hanburys Ltd., the furazolidone by A. J. White Ltd., the dihydrostreptomycin by Glaxo Laboratories Ltd., the sulphamerazine by May & Baker Ltd., and the terramycin by Pfizer Ltd.

REFERENCES

Alberts, J. O. (1950). *Amer. J. vet. Res.*, **11**, 421.
Boney, W. A. (1951). *Ibid.*, **12**, 134.
Cooper (1954). *Personal communication.*
Cox, F. B., and Craig, F. R. (1952). *J. Amer. vet. med. Ass.*, **120**, 293.
Evans, R. J. (1948). *J. Hyg., Camb.*, **46**, 422.
Grumbles, L. C., Wills, F. K., and Boney, W. A. (1954). *J. Amer. vet. med. Ass.*, **124**, 217.
Hall, W. J., MacDonald, A. D., and Legenhausen, D. H. (1949). *Poult. Sci.* **28**, 789.
Hammond, J. C. (1945). *Ibid.*, **24**, 382.
Holtman, D. F., and Fisher, G. (1947). *Ibid.*, **26**, 478.
Moore, E. N. (1946). *Ibid.*, **25**, 307.
Prier, J. E. and Alberts, J. O. (1951). *Amer. J. vet. Res.*, **12**, 156.
Smith, H. Williams. (1954a). *J. comp. Path.*, **64**, 225; (1954b). *Vet. Rec.*, **66**, 215; (1955). *J. comp. Path.*, **65**, 37.

[*Received for publication, July 8th,* 1954]

J. Comp. Path. 1955. Vol. 65.

THE BLOOD GROUPS OF THE PIG

I. The Interaction of Pig Red Cells of Group A and the Naturally Occurring A Iso-Antibody in the Serum of Pigs of Blood Group O

By

Ruth Saison* and R. F. W. Goodwin

Department of Veterinary Clinical Studies, Madingley Road, Cambridge

and

R. R. A. Coombs

Department of Pathology, Cambridge

INTRODUCTION

Recent reports on the occurrence of haemolytic disease in the new-born pig (Buxton and Brooksbank, 1953 a, b; Szent Iványi and Szabó, 1953; Doll and Brown, 1954) have lent a fresh impetus to the study of the blood groups in this species. Fortunately, much has been learnt of the nature and serological properties of natural and immune iso-antibodies since the earlier work of Szymanowski, Stetkiewicz and Wachler (1926), Kaempffer (1932) and Hardt (1937) and, as a result, more reliable and sensitive techniques are now available for the re-investigation of this subject.

As a preliminary study, and as part of a wider investigation now in progress on the blood groups of pigs, we chose to examine the interaction of group A cells and the naturally occurring A iso-antibody. To procure sera containing the naturally occurring A iso-antibody uncontaminated with other immune red cell iso-antibodies, pigs were reared and maintained without inoculation with crystal violet swine fever vaccine. This was necessary as, theoretically, this vaccine was considered capable of stimulating the production of red cell iso-antibodies. The sera studied were from pigs of six to seven months of age, at which age, according to the earlier workers, the naturally occurring anti-A is usually fully developed.

Once sera containing antibodies characteristic of anti-A were recognised, the ability of different serological techniques to show the interaction was appraised. The methods investigated were direct agglutination, indirect anti-globulin sensitization test (Coombs, Mourant and Race, 1945), agglutination of cells treated with trypsin (Morton and Pickles, 1951) and immune haemolysis using either guinea-pig or pig complement. The most reliable method was found to be the anti-globulin sensitization test. The agglutination of cells treated with trypsin appeared reliable but was less sensitive. Direct agglutination could not be relied upon to demonstrate the presence of anti-A.

*From the Ontario Veterinary College, Canada.

METHODS

Bleeding of pigs. Blood was obtained by inserting a needle into an ear vein.

Red blood cells. Blood was collected into bottles containing acid-citrate-dextrose solution as used in human blood transfusion. About two volumes of blood were added for each volume of citrate solution. The citrated blood was stored at $+4^0$C., and used for periods up to one week. Each day as required a portion of the cells was washed three times in saline (0.9 per cent and buffered at pH 7.2 with phosphate buffer) before being used as a 2 per cent suspension for serological tests.

Sera. The sera were separated from clotted blood and stored at -20^0C. without the addition of any chemical preservative. Sera were used both unheated and after heating at 56^0C. for half an hour.

Serological tests

Direct agglutination. One drop of a 2 per cent suspension of washed cells was added to one drop of serial dilutions of the serum in Rh tubes.* The contents of the tubes were mixed and the cells allowed to sediment at room temperature. The deposited cells were examined with the aid of a simple lens and also by removing with a Pasteur pipette and examining them on a glass slide under a microscope using the 2/3 objective.

Indirect anti-globulin sensitization test. The test was performed as described by Coombs, Mourant and Race (1945) except that a rabbit anti-pig globulin serum was used to test for sensitization. The primary incubation was carried out at room temperature, as in the direct agglutination test, while the secondary incubation with the anti-globulin serum was carried out at 37^0C.

The anti-globulin serum was prepared by injecting rabbits with alum-precipitated pig serum. The serum obtained after eight intramuscular injections of 4 ml. was heat inactivated and absorbed with seven times washed pig cells till free of agglutinins for these. This serum then had no demonstrable action on washed normal pig cells, but agglutinated strongly a washed suspension of pig cells sensitized with pig iso-antibody. The serum was used at a dilution of 1 in 10.

Agglutination tests using cells treated with trypsin. The procedure followed closely that described by Morton and Pickles (1951). Two ml. of a 2 per cent suspension of washed pig cells were mixed with 2 ml. of a 1 per cent solution of trypsin (B.D.H. commercial trypsin) in 0.15 M phosphate buffer pH 7.2. After one hour at 37^0C., the cells were deposited by centrifugation, washed once with saline, and then resuspended in saline to give a 2 per cent suspension: 0.1 ml. volumes of this suspension of cells treated with trypsin were then added to 0.1 ml. volumes of varying dilutions of serum. After one hour at 37^0C. or room temperature, the tubes were centrifuged lightly for 20 seconds and the deposited cells examined under the microscope for agglutination.

Immune haemolysis tests. The heat-inactivated pig sera were titrated in 0.1 ml. amounts and 0.1 ml. of a 1 per cent suspension of pig cells added. Then 0.1 ml. of either fresh pig or guinea-pig serum was added to each tube as a source of complement. The pig serum was from a group A pig

*An Rh tube is a rimless glass test tube 7 × 50 mm.

and was used at a dilution of 1 in 5. The guinea-pig serum had previously been absorbed in the cold with pig cells, to remove naturally occurring antibodies to pig cells, and was used at a dilution of 1 in 10. The tests were incubated at 37°C. for half an hour, after which the tubes were examined for haemolysis. The activity of the complements was controlled using bovine red cells sensitized with rabbit antibody.

RESULTS

Comparison of four different serological techniques as a means of demonstrating the interaction of pig group A cells and the naturally occurring iso-antibody to the A antigen

The techniques compared were direct agglutination, indirect anti-globulin sensitization test, agglutination of cells treated with trypsin and immune haemolysis. In the first place some anti-A sera were identified using the ordinary agglutination reaction by the fact that the antibody was naturally occurring in pigs of group O, and by the fact that it was absorbed by human group A cells but not by human group O cells.

TABLE 1

COMPARISON OF FOUR SEROLOGICAL TECHNIQUES

Comparison of four techniques for the demonstration of the interaction of Pig group A cells and the naturally occurring A iso-antibody.

Anti-A serum	*A cells*	*Technique used to demonstrate interaction*	*Titre of anti-A demonstrated*
M 16	Pig 202	Direct agglutination	1
,,	,,	Indirect sensitization test	1024
,,	,,	Agglutination of trypsin—treated cells	64
,,	,,	Immune haemolysis (pig complement)	—
,,	,,	Immune haemolysis (guinea-pig complement)	—
M 46	,,	Direct agglutination	—
,,	,,	Indirect sensitization test	4
,,	,,	Agglutination of trypsin—treated cells	(1)

( ) Weak agglutination.

— No agglutination or haemolysis.

With pig cells of group O the two sera gave negative results by all five tests.

For the comparison of the four serological techniques in this system, a powerful (M16) and a weak (M46) anti-A serum were chosen and their reactions against the cells of a particular group A pig studied. The results of the experiment are shown in Table 1. Whereas with serum M16 the anti-A could be demonstrated by the ordinary agglutination test only with the undiluted serum, its presence could be shown by the anti-globulin test in serum diluted 1 in 1024. Treatment of the cells with trypsin allowed a satisfactory demonstration of the antibody, but the method was not as sensitive as the anti-globulin test. With the serum M46 the anti-A could be demonstrated unequivocally only by the anti-globulin test. Neither pig nor guinea-pig complement was able to effect haemolysis of the sensitized cells.

A possible reason for the poor reaction with the ordinary agglutination technique is suggested under the section on cross-specificity.

Comparison of the direct agglutination reaction with the indirect anti-globulin sensitization test in the titration of anti-A in the sera of nine group O pigs against the cells of seven group A pigs

The tests were set up as described under Methods and the results (Table 2) were similar whether the sera were used unheated or after being heated at 56°C. for half an hour. All sera gave negative results against group O cells with both tests. The unsuitability of the ordinary agglutination reaction for the demonstration of the interaction is clear. For instance, using this reaction alone serum M46 appeared to contain no anti-A, while most of the other sera, even when undiluted, failed to agglutinate every group A cell in the panel.

Cross-specificity between the anti-A in pig sera and the A antigen on human red cells

Szymanowski, *et. al.* (1926) showed that anti-A in pig serum was specifically absorbed by human group A cells and was not absorbed by human group O or group B cells. In our experiments, the anti-A in the sera of pig M 16 and M 17 was capable of being completely absorbed by human group A cells. A comparable absorption with human group O cells left the antibody unaltered.

The serum of pig M 16, absorbed free of antibodies to human group O cells, was still capable of sensitizing pig group A cells to the original serum titre and of agglutinating directly human group A cells up to a serum dilution of 1 in 128. The pig anti-A serum, which when diluted 1 in 2 can be detected on pig group A cells only by the anti-globulin sensitization test or after digestion of the cells with trypsin, is capable, nevertheless, of directly agglutinating human group A cells. Thus the reason the anti-A in pig serum fails to agglutinate directly pig group A cells appears not to be an expression of the 'incompleteness' of the antibody but rather to be due to the nature of the antigen or its situation on the pig red cell membrane.

TABLE 2

AGGLUTINATION REACTIONS

Comparison of the direct agglutination reaction with the indirect anti-globulin sensitization test in the titration of anti-A in the sera of nine group O pigs against the cells of seven group A pigs.

Sera of group O pigs containing anti-A	*Direct agglutination*							*Indirect anti-globulin sensitization test*						
	Group A cells							*Group A cells*						
	C202	C	S102	M38	M41	M43	B1	C202	C	S102	M38	M41	M43	B1
M 16	±	1	1	?	±	∓	—	1024	1024	1024	512	1024	512	2048
M 17	1	±	1	±	1	1	±	32	32	64	32	32	32	128
M 21	?	?	?	±	±	±	—	8	8	16	4	8	4	32
M 26	1	2	2	2	2	2	?	32	32	128	32	32	64	64
M 28	2	1	2	2	2	2		8	16	16	16	16	8	
M 34	1	1	±	?	?	±		8	16	16	16	32	8	
M 42	1	1	1	2	1	1		16	64	32	16	32	32	
M 46	—	—	—	—	—	—		4	16	8	4	4	4	
M 50	1	1	1	1	±	?		16	32	32	32	32	32	

Figures 1 to 2048 indicate the titre of serum showing definite agglutination.

± = Weak agglutination with undiluted serum.

? = Doubtful agglutination with undiluted serum.

— = No agglutination with undiluted serum.

All sera gave a negative result against group O cells by both reactions.

Injection of a group O pig with A substance

Before the high level of naturally occurring anti-A in the serum of pig M 16 was discovered, it was considered worth while to attempt to raise the anti-A in the sera of group O pigs by injection of pig gastric mucoid possessing A specificity. It was hoped that this substance might prove antigenic in pigs, as in man, and that besides raising the serum level of anti-A, it would also stimulate the production of directly agglutinating antibody.

Professor W. T. J. Morgan kindly supplied us with purified pig gastric mucoid possessing A activity (Morgan and King, 1943). A 1 per cent. solution of this A substance, further diluted over 300,000 times, was capable of inhibiting in vitro the combining capacity of a 1 in 20 dilution of pig anti-A serum in which the sensitizing titre was over 1024. A solution was made containing 27 mg./ml. and this was stored at $+4°$C. with toluene as preservative. A group O pig (M 17) six months of age, which had received no swine fever vaccine and had an existing anti-A titre of 64 received 10 bi-weekly intramuscular injections of 1 ml. of mucoid solution, a total of 270 mg. A serum sample was obtained 10 days after the fourth injection and again 8, 22 and 42 days after the tenth injection. The levels of anti-A in the serum samples are shown in Table 3. No rise in antibody level was demonstrated in this pig. The fall in the existing level of anti-A indicates perhaps that too big a dose was injected and neutralization of the antibody occurred. However, even 22 and 42 days after the last injection, the antibody level showed no indication of rising.

TABLE 3

INJECTION OF PIG GASTRIC MUCOID

Anti-A in the serum of a group O pig before and after injections of pig gastric mucoid possessing A activity.

Serum samples	*Titre of anti-A demonstrable by two methods*	
	Direct agglutination	*Anti-globulin sensitization test*
Pre-injection	0	64
10 days after 4th injection	4	64
8 days after 10th injection	0	2
22 days after 10th injection	0	2
42 days after 10th injection	0	0

0 = Less than 2.

DISCUSSION

Previous workers using the ordinary agglutination test have had difficulty in obtaining trustworthy reactions between the natural anti-A in pig sera and pig group A cells. The present study gives an explanation for this difficulty, as it shows the unsuitability of the ordinary agglutination test for this reaction. It is suggested that the reason the direct agglutination reaction is unsuited lies probably in the particular situation of the A antigen on pig red cells or to some other property of the cells themselves. Using anti-A pig sera, group A cells can be recognised unequivocally by the indirect anti-globulin sensitization test, and vice versa this test is well suited for measuring the titre of anti-A in pig sera.

Szymanowski, *et. al.* (1926) and Kaempffer (1932), who used only the ordinary agglutination test for their studies, stated that pigs may be divided into three main groups according to the serological characteristics of their blood.

Group I A antigen on the cells and no natural anti-A in the serum.
Group II No A-antigen on the cells but anti-A in the serum.
Group III No A-antigen on the cells and no anti-A in the serum.

In the light of the present study, many animals previously placed in group III seem to belong to group II. However, in our experience in tests on about 50 group O pigs, one animal was encountered which at 2 months of age had no demonstrable anti-A in its serum. At 4 months, the antibody was just demonstrable by the anti-globulin test.

CONCLUSIONS

The direct agglutination reaction is unsuited for the unequivocal recognition of pig group A cells with the naturally occurring A iso-antibody. The anti-globulin sensitization test is a reliable method for detecting the interaction. The previous treatment of group A cells with trypsin allows them to be agglutinated by the A iso-antibody, but this method is not as sensitive as the anti-globulin sensitization test.

Many animals previously grouped as O_0 (no A antigen on the cells and no anti-A in the serum) probably in fact belonged to group $O\alpha$ (no A antigen on the cells but containing anti-A in the serum).

Injections of large doses of pig gastric mucoid possessing A activity into a single pig of group $O\alpha$ did not raise the level of anti-A in the serum; in fact the existing level of anti-A was suppressed.

ACKNOWLEDGMENTS

The authors wish to thank the Agricultural Research Council for a grant towards the cost of this work. One of us (R.R.A.C.) wishes to express his appreciation to the Agricultural Research Council and Nuffield Foundation for a joint personal grant. The authors would also like to

thank Professor L. P. Pugh for his efforts in getting this work started and his interest in its progress. Finally, thanks are due to Professor W. T. J. Morgan for supplying the purified pig gastric mucoid.

REFERENCES

Buxton, J. C., and Brooksbank, N. H. (1953a). *Vet. Rec.*, **65**, 287; (1953b). *Nature, Lond.*, **172**, 355.
Coombs, R. R. A., Mourant, A. E., and Race, R. R. (1945). *Brit. J. exp. Path.*, **26**, 255.
Doll, E. R., and Brown, R. G. (1954). *Cornell Vet.*, **44**, 86.
Hardt, O. (1937). *Z. Rassenphysiol.*, **9**, 178.
Kaempffer, A. (1932). *Z. indukt. Abstamm.-u. VerebLehre*, **61**, 261.
Morgan, W. T. J., and King, H. K. (1943). *Biochem.*, **37**, 640.
Morton, J. A., and Pickles, M. M. (1951). *J. clin. Path.*, **4**, 189.
Szent Iványi, T., and Szabó, St. (1953). *Acta Vet. hung.*, **3**, Fasc. 1, 75.
Szymanowski, Z., Stetkiewicz, St., and Wachler, B. (1926). *C. R. Soc. Biol., Paris*, **94**, 204.

[*Received for publication, June 21st,* 1954]

J. Comp. Path. 1955. Vol. 65.

THE BLOOD GROUPS OF THE PIG

II Red Cell Iso-antibodies in the Sera of Pigs Injected with Crystal Violet Swine Fever Vaccine

By

R. F. W. Goodwin and Ruth Saison*

Department of Veterinary Clinical Studies, Madingley Road, Cambridge

and

R. R. A. Coombs

Department of Pathology, Cambridge

INTRODUCTION

Haemolytic disease of new-born pigs was first definitely diagnosed in this country by Buxton and Brookshank (1953, a and b) although, in retrospect, it is probable that the syndrome had been observed clinically before this date. In any discussion on the aetiology of this condition, therefore, it is safer to assume that the cases studied by Buxton and Brooksbank were not the first to occur and, if the impressions of Kershaw (1950) and others were correct, the disease has been apparent for 4 years or more.

Most of the affected sows studied by Buxton and Brooksbank were transferred to Cambridge in 1953 to allow a wider study of the disease, and for this purpose investigations were initiated along two main lines. First, the disease in the piglet has been studied from the clinical, haematological, histopathological and serological aspects. Second, a survey has been made into the incidence and distribution of red cell iso-antibodies on a herd basis with the aim of understanding the factors that determine the pattern of the disease within the pig population. It is with this latter work that the results described in this and further papers in this series are concerned.

From the existing knowledge on haemolytic disease, it was clear that pigs could become sensitized from the injection of material containing incompatible red-cell antigens and that, on theoretical grounds, crystal violet swine fever vaccine could provide such material, for it is prepared from the blood of pigs bled at the height of virus infection. Consequently, our first investigations concerned the ability of this vaccine to stimulate the production of red cell iso-antibodies.

The experiments designed to demonstrate these immunological side-effects of the vaccine were somewhat artificial in that the injections were made more frequently than would be the case in a normal swine fever immunisation programme. It had been intended, therefore, that they would be modified later until they conformed to the practice of vaccination in the field. However, once it was

*From the Ontario Veterinary College, Canada

apparent from this preliminary study that iso-antibodies could be readily produced by injections of vaccine, it seemed preferable to defer further work on such small groups of animals and determine, instead, whether the distribution of iso-antibodies among pigs generally was related to a history of vaccination.

The results presented below illustrate not only the production of red cell iso-antibodies by experimental vaccination, but demonstrate also that, among pigs maintained under ordinary farm conditions, there is a marked difference between vaccinated and unvaccinated animals in both the occurrence and the concentration of serum iso-antibodies.

MATERIALS AND METHODS

Pigs. The young pigs used for the vaccination experiments were reared on the Veterinary School Farm and were all out of a group of Essex sows by one Large White boar. The Essex sows were sired by the same Essex boar and they originated from 2 litters whose respective dams were litter sisters.

Sera from 21 of these young pigs, taken before vaccination, provided some of the control samples. The remaining control samples of serum were obtained almost entirely from 3 farms where vaccination had not been generally practised and where, as a result, most of the adult stock were unvaccinated. In a few instances, unvaccinated pigs were encountered when bleeding the vaccinated herds and samples from these were added to the control group.

There is an important difference between the controls reared on the School farm and those obtained in the field. In the first case, the pigs were certainly unvaccinated but in the second, it was necessary to rely on the owners' statements and, although every effort was made to check this information by reference to an owner's veterinary surgeon and to the previous owners of any pigs that had been brought in, it is always possible that in these circumstances the control group could include a few vaccinated pigs. Any of the control pigs, however, that had an obviously uncertain vaccination history have not been included in the final results.

Sera from pigs vaccinated in the field were first obtained from herds vaccinated by practising veterinary surgeons at the normal request of their clients. Our attention had been directed to these herds, however, by the fact that one or more sows in each herd had produced a litter that suffered from haemolytic disease. To counterbalance these selected herds, therefore, the remainder of the vaccinated pigs were obtained from herds in which no clinical history of haemolytic disease existed. Five of the seven herds so chosen were particularly suitable, as they were directly supervised by veterinary surgeons on the staff of the School and any clinical symptoms of haemolytic disease should have been more readily observed.

For the vaccinated group as a whole, the vaccination histories cannot be completely reliable but they should be substantially correct.

Blood samples. The pigs were bled by allowing blood to drip freely from a needle inserted in an ear vein. Two separate samples were taken from each pig: one of clotted blood for serum and the other of about 2 volumes of blood into 1 volume of acid-citrate-dextrose solution. Generally these samples from the field were stored overnight and handled the following day in the laboratory. In this case, the clotted blood stood at room temperature while the citrated samples were kept at +4°C.

Serological tests. The preparation of a washed suspension of red cells for testing and the performance of the direct agglutination and indirect anti-globulin sensitization tests were carried out as described in our earlier paper on the interaction of pig A cells and the corresponding naturally occurring antibody (Saison, Goodwin and Coombs, 1955). In the present tests, however, the primary incubation of the cells and sera was carried out at 37°C. instead of at room temperature, as the antibodies being investigated were of the immune type. In addition, the pig sera were used unheated.

All the sera were examined with O cells, to exclude reactions due to anti-A. The A-O grouping of cells was performed using a 1/10 dilution of an anti-A pig serum of titre 1024, and the interaction was detected by an anti-globulin serum (Saison, *et. al.*, 1955).

RESULTS

Optimal Red Cells for Testing Pig Sera for Red Cell Iso-antibodies

Before attempting to demonstrate red cell immune iso-antibodies in the sera of pigs, it was necessary to find a source of red cells containing as many of the pig blood group antigens as possible.

As yet, there exists no body of information on pig blood groups other than that on the A - O system, but from the knowledge of blood groups in other animal species and man it is very unlikely that any one cell could contain more than a proportion of the antigens occurring in the species. Thus to cover all the antigen-antibody systems, supposing for a moment that they could be recognised, probably many testing cells would have to be used. However, the object of the present work was not to measure and characterize all the red cell iso-antibodies in the sera examined, but only to examine the possibility that red cell iso-antibodies could be produced to an extent that might be dangerous in pigs vaccinated with crystal violet swine fever vaccine.

Clear evidence of this production of iso-antibodies has been obtained by the examination of each serum sample against two types of cells: (a) a standard multiple-antigen cell from a group O pig (57) and (b) the cells of chance antigenic structure taken from the

appropriate breeding boar of the herd concerned. To avoid confusing the naturally occurring anti-A in the sera of pigs of group O with immune iso-antibodies, cells of only group O pigs were used for testing. Fortunately, all the local breeding boars were of group O.

The cells of pig 57 were selected empirically as being very suitable for measuring iso-antibodies. They were found in the first place by testing a panel of group O cells against the sera of six sows that had had litters affected with haemolytic disease. The cells of this pig recognised the antibodies in all cases and were very sensitive in their ability to detect them. Preliminary tests had shown that these six sera possessed a complicated mixture of antibodies and thus the cells of pig 57 must have possessed a correspondingly varied antigenic structure. The suitability of this cell has been amply borne out by subsequent tests.

Demonstration of the Rise of Red Cell Iso-antibodies, other than anti-A, in the Sera of Pigs Injected with Crystal Violet Swine-fever Vaccine

In the first experiment, four unvaccinated pigs of 5 to 7 months of age were bled for a pre-injection sample of serum and then injected with 5 ml. doses of crystal violet vaccine Batch 101* at fortnightly intervals for a total of six injections. Weekly blood samples were taken and the sera examined for antibodies reactive against cells 57 and the cells of the boar on the School farm (Madingley boar).

In the second experiment, a further four unvaccinated pigs aged 5 to 7 months were bled for a pre-injection sample of serum and then injected with 5 ml. doses of crystal violet vaccine, Batch 105, at fortnightly intervals for a total of four injections. Weekly blood samples were again taken and the sera examined for antibodies reactive against cells 57. The third experiment was comparable with the second in all ways except that Batch 106 of the vaccine was used.

The sera were examined for iso-antibodies by the indirect anti-globulin sensitization test to be sure of detecting incomplete as well as complete antibodies. In order to see whether in fact the extra work involved in performing the anti-globulin test was justified, the much simpler direct agglutination test was also carried out on each serum sample so that the two results could be compared. The results of all the serological tests are summarised in Tables 1, 2, 3 and 4, where it will be seen that the demonstration by the anti-globulin sensitization test of antibodies not detected by direct agglutination amply justified the use of the former test.

In the first experiment (Tables 1 and 2), three pigs responded to the challenge of repeated injections of vaccine by producing red cell iso-antibodies detectable by cells 57. In two of the pigs (25 and 29), the antibody content reached a level that was possibly dangerous, for clinical cases of haemolytic disease of the new born

*All samples of crystal violet swine fever vaccine used in this study were kindly supplied by The Director, Veterinary Laboratory, Ministry of Agriculture, Weybridge.

have been observed in the field in which the serum antibody titre of the sow has been as low as 256 or even 128.

Testing the same sera with the red cells of the Madingley boar, instead of pig 57, showed that the former cells failed to recognise these high titred iso-antibodies. Interestingly enough, however, the boar's cells recognised an antibody in serum 12 that cells 57 failed to detect.

TABLE 1

Red cell iso-antibodies in the sera of four pigs injected with crystal violet swine fever vaccine (Batch 101) as shown by tests with the cells of pig 57.

Serum samples in relation to injections	*Pig* 12 (*Group* O)	*Pig* 15 (*Group* O)	*Pig* 25 (*Group* O)	*Pig* 29 (*Group* A)
	Serum antibody titres			
7.12.53. Pre-injection	0 (0)	0 (0)	0 (0)	0 (0)
7.12.53. 1st injection				
14.12.53.	0 (0)	0 (0)	0 (0)	0 (0)
21.12.53.	0 (0)	0 (0)	0 (0)	0 (0)
21.12.53. 2nd injection				
4.1.54.	0 (0)	0 (0)	2 (1)	0 (0)
4.1.54. 3rd injection				
11.1.54.	<2(<2)	8(<2)	16 (4)	1 (0)
18.1.54.	<2(<2)	8(<2)	32 (4)	2 (1)
18.1.54. 4th injection				
28.1.54.	<2(<2)	32(<2)	256 (32)	128 (4)
1.2.54.	<2(<2)	32(<2)	256 (32)	128 (8)
1.2.54. 5th injection				
8.2.54.	<2(<2)	32(<2)	256 (64)	256(32)
15.2.54.		16(<2)	512 (64)	>256(32)
15.2.54. 6th injection				
24.2.54.	<2(<2)	16(<2)	2048(128)	>256(32)
1.3.54.	<2(<2)	16 (1)	2048(64)	512(64)
6.5.54.			128 (16)	
2.6.54.		2 (0)	64 (16)	64 (16)

The figures without brackets indicate the serum titres demonstrated by the anti-globulin sensitization test. The figures within the brackets indicate the titres demonstrated by direct agglutination.

The conclusions from experiments 2 and 3 (Tables 3 and 4) are similar to those from the first experiment. In these two later experiments, only four injections of vaccine were given and again approximately half of the pigs produced iso-antibodies to a titre of 256 or 512.

TABLE 2

Red cell iso-antibodies in the sera of four pigs injected with crystal violet swine fever vaccine (Batch 101) as shown by tests with the cells of the Madingley boar.

Serum samples in relation to injections	*Pig* 12 (*Group* O)	*Pig* 15 (*Group* O)	*Pig* 25 (*Group* O)	*Pig* 29 (*Group* A)
	Serum antibody titres			
7.12.53. Pre-injection	0	0	0	0
7.12.53. 1st injection				
14.12.53.	0	0	0	0
21.12.53.	0	0	0	1
21.12.53. 2nd injection				
4.1.54.	0	0	0	4
4.1.54. 3rd injection				
11.1.54.	0	0	1	4
18.1.54.	1	0	4	4(0)
18.1.54. 4th injection				
28.1.54.	2	1	8	4(0)
1.2.54.	4	1	8	4(0)
1.2.54. 5th injection				
8.2.54.	4(<2)	8(<2)	32 (0)	4(0)
15.2.54.	8	16(<2)	32 (0)	4(0)
15.2.54. 6th injection				
24.2.54.	8(<2)	16(<2)	64 (0)	4(0)
1.3.54.	16(<2)	16(<2)	64 (0)	4(0)

Governing the failure to produce red cell iso-antibodies, there is the constitutional inability of a pig of a certain blood group genotype to produce the corresponding antibodies as well as the other unknown factors that influence antibody production. In experiment 2 (Table 3), as a result of testing serum 41 against the cells of pig 6, it was shown that the antibody or antibodies in serum 6 were of a different specificity from those in serum 41. Also pig 22, because its cells reacted with the antibodies in serum 41, could not, on account of its own blood group constitution, produce antibodies similar to those found in the serum of pig 41. On the other hand, no such constitutional factor could be shown that might have prevented pig 22 from producing an antibody of similar specificity to the antibody produced by pig 6.

From these experiments, it was shown that crystal violet vaccine was capable of stimulating red cell iso-antibodies in pigs. However, as has been explained already, the number and frequency of these experimental injections constituted an unusually intensive antigenic stimulation, and the experimental programme was further removed from the normal practice in the field by using the same

Table 3

Red cell iso-antibodies in the sera of four pigs injected with crystal violet swine fever vaccine (Batch 105) as shown by tests with the cells of pig No. 57

Serum samples in relation to injections	*Pig 6 (Group O)*	*Pig 22 (Group O)*	*Pig 38 (Group A)*	*Pig 41 (Group A)*
	Serum antibody titres			
8.3.54. Pre-injection bleed	0 (0)	0 (0)	0 (0)	0 (0)
8.3.54. 1st injection				
15.3.54.	0 (0)	0 (0)	0 (0)	0 (0)
22.3.54.	1 (0)	0 (0)	0 (0)	0 (0)
22.3.54. 2nd injection				
31.3.54.	>128 (64)	0 (0)	0 (0)	32 (32)
6.4.54.	512 (64)	0 (0)	<2(<2)	32 (32)
6.4.54. 3rd injection				
12.4.54.	512 (64)	0 (0)	<2(<2)	64 (32)
20.4.54.	512(128)	0 (0)	<2(<2)	128 (16)
20.4.54. 4th injection				
26.4.54.	512(128)	1 (1)	4(<2)	128 (16)
3.5.54.	512(128)	1 (1)	4(<2)	64 (8)
10.5.54.	>512(128)	1 (1)	4(<2)	64 (8)
17.5.54.	>512(128)	0 (0)	4(<2)	32 (8)

Table 4

Red cell iso-antibodies in the sera of four pigs injected with crystal violet swine fever vaccine (Batch 106) as shown by tests with the cells of pig No. 57

Serum samples in relation to injections	*Pig 35 (Group O)*	*Pig 40 (Group O)*	*Pig 43 (Group A)*	*Pig 48 (Group O)*
	Serum antibody titres			
8.3.54. Pre-injection bleed	0 (0)	0 (0)	0 (0)	0 (0)
8.3.54. 1st injection				
15.3.54.	0 (0)	0 (0)	0 (0)	0 (0)
22.3.54.	0 (0)	0 (0)	0 (0)	0 (0)
22.3.54. 2nd injection				
31.3.54.	0 (0)	64 (4)	0 (0)	0 (0)
6.4.54.	8 (2)	64 (8)	<2(<2)	<2(<2)
6.4.54. 3rd injection				
12.4.54.	32 (2)	128 (8)	16(<2)	<2(<2)
20.4.54.	128 (2)	256 (8)	32 (4)	<2(<2)
20.4.54. 4th injection				
26.4.54.	256 (4)	>256 (16)	32 (4)	2(<2)
3.5.54.	256 (4)	256 (16)	32 (4)	2(<2)
10.5.54.	256 (4)	>256 (16)	32 (4)	2(<2)
17.5.54.	>256 (4)	256 (8)	32 (4)	<2(<2)

batch of vaccine throughout each experiment. Thus our next consideration was whether the vaccine, as administered for the routine prophylaxis of swine fever, could likewise stimulate the production of red cell iso-antibodies and, if this were so, whether it could do so to an extent that might prejudice health by rendering sows liable to have litters affected with haemolytic disaese.

The results of this enquiry are presented in the following section.

Red Cell Iso-antibodies, other than anti-A, in the Sera of Pigs Injected in the Field with Crystal Violet Swine-fever Vaccine and of Uninjected Pigs.

A total of 152 vaccinated pigs, excluding the breeding boars, were bled for this part of the investigation. Of this number 52, including 5 clinical cases, came from 3 herds where haemolytic disease had been diagnosed. A further 8 samples were obtained from isolated clinical cases where other pigs in the herd were not bled. Thus a total of 13 clinical cases are included in the vaccinated group. Concerning the vaccinated pigs from herds with no history of haemolytic disease, a total of 92 samples were obtained from 7 herds, and 80 of these samples came from the 5 herds supervised by the School.

Fig. 1

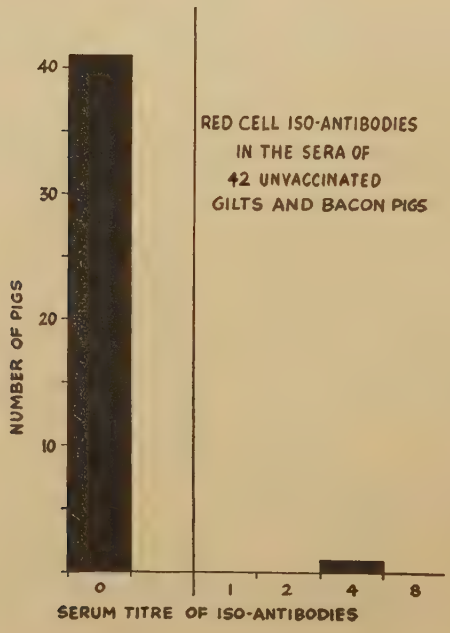


Fig. 2

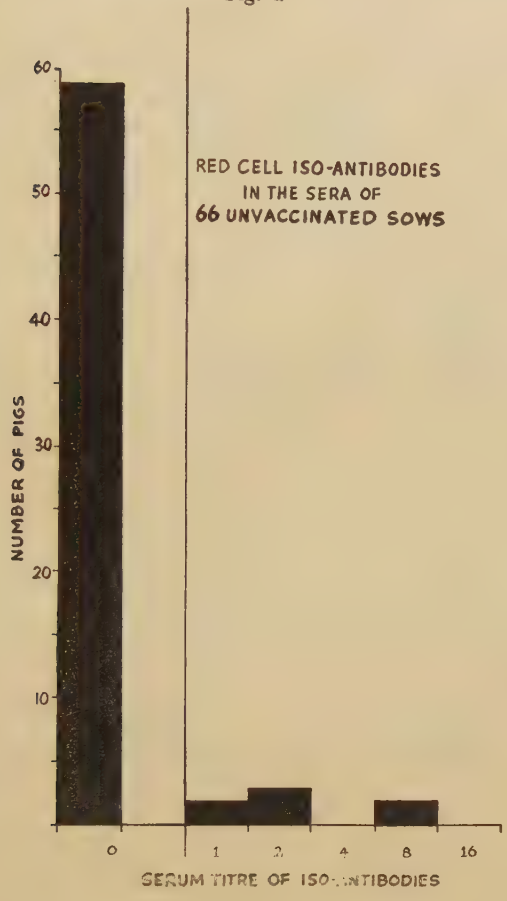

RED CELL ISO-ANTIBODIES
IN THE SERA OF
66 UNVACCINATED SOWS
NUMBER OF PIGS
60
50
40
30
20
10
0
1
2
4
8
16
SERUM TITRE OF ISO-ANTIBODIES

Fig. 3

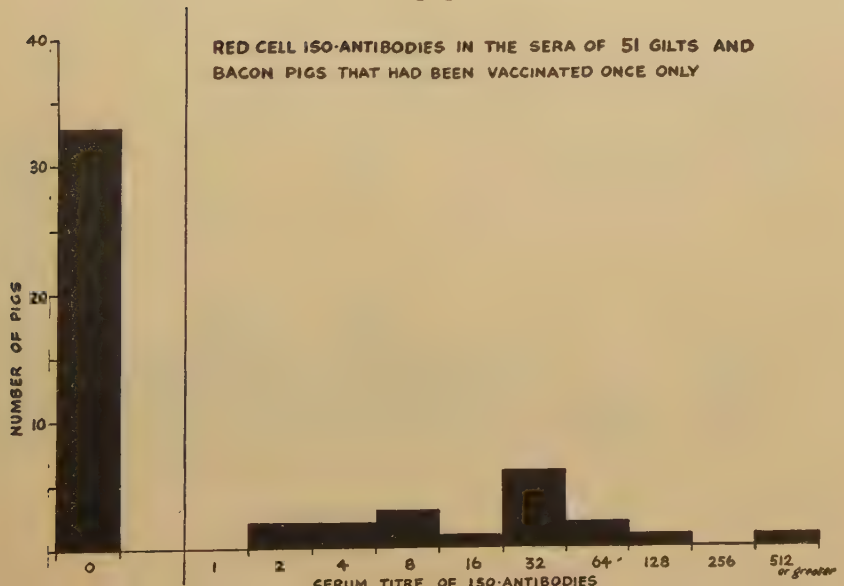

RED CELL ISO-ANTIBODIES IN THE SERA OF 51 GILTS AND
BACON PIGS THAT HAD BEEN VACCINATED ONCE ONLY
NUMBER OF PIGS
40
30
20
10
0
1
2
4
8
16
32
64
128
256
512
or greater
SERUM TITRE OF ISO-ANTIBODIES

Fig. 4

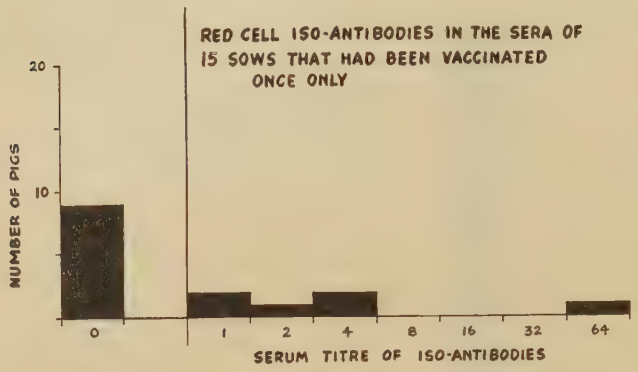


Fig. 5

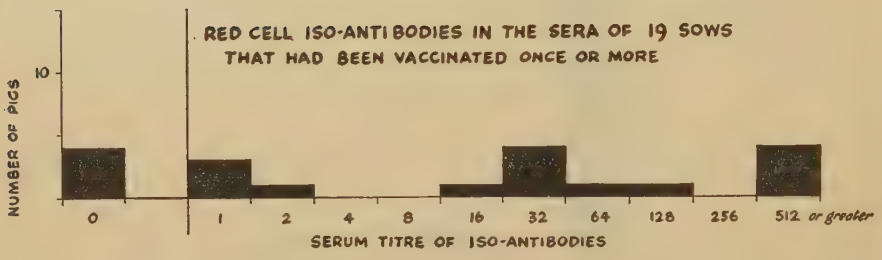
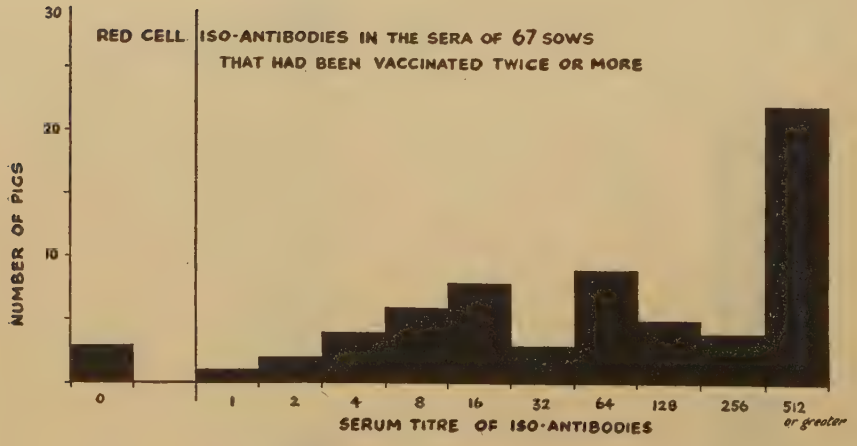


Fig. 6



The number of control samples from unvaccinated pigs, again excluding the boars, is 108. These include the 21 of our own pigs, plus 84 pigs from 3 other herds. The remainder (3) were isolated samples from an otherwise vaccinated herd. Within this control group, 67 animals were said to have farrowed at least once, and of these, 34 were reported to have had 2 or more litters, while 21 were recorded as having farrowed 3 times or more. We are aware of the limited number of controls and would have preferred more, particularly from a greater variety of unvaccinated herds, but there were special difficulties in obtaining samples from unvaccinated animals.

The sera from the vaccinated and unvaccinated pigs were examined unheated by the direct agglutination and indirect anti-globulin sensitization test for antibodies reactive against cells 57 and the cells of the respective local boar. The results are recorded in the form of histograms, which show the titre of antibody detected by the anti-globulin test against cells 57 or those of the local boar, whichever titre was the higher.

Without any detailed analysis of the figures, it is clear that prophylactic vaccination, as carried out in the field, engenders the production of red cell iso-antibodies. In sows vaccinated more than once the proportion of animals with high and possibly dangerous levels of antibody becomes most significant.

The explanation of the occasional low titre of antibody, 8 being the highest, in unvaccinated pigs is uncertain. It may represent either normal iso-antibodies or immune antibodies produced in response to some as yet unknown stimulus.

DISCUSSION

It has been established that crystal violet vaccine can stimulate the production of red cell iso-antibodies in pigs. It has also been shown that within the pig population at large, those animals with detectable levels of serum iso-antibodies are confined almost exclusively to vaccinated herds. Furthermore, high levels of serum iso-antibodies are common among vaccinated pigs, whereas the highest level encountered among unvaccinated animals was represented by a titre of 8. It may be deduced, therefore, that the use of crystal violet vaccine has been responsible for the sensitization of breeding gilts and sows in the field, and has set the stage for the production of haemolytic anaemia in the baby pig.

The extent to which such sensitization has occurred cannot be legitimately assessed from the pattern of iso-antibodies in our total sample of vaccinated pigs because the animals were largely of one breed and they were further selected by the inclusion of an unusually large proportion of pigs from herds in which symptoms of haemolytic disease had been observed clinically. However, the nature of our survey does not preclude a tentative answer to this question, and

some indication of the effect of vaccination can be obtained from the results relating to the five supervised herds that had no clinical history of haemolytic disease.

The number of breeding females within these five herds was 54 and, of these, 30 per cent had an iso-antibody titre of 128 or more.* The incidence of high titres among these pigs may have been exaggerated by the fact that virtually all the animals were of one breed. In addition, the vaccination programme was unusual—in so far as it was regular and in some cases biannual—which may, again, have favoured the incidence of high titres. Despite these qualifications, however, and after allowing for the small number of animals involved, it does seem most probable that, among the general breeding stock of vaccinated herds, the incidence of iso-antibodies at a titre of 128 or more is considerable.

We are thus confronted with a situation in which large numbers of breeding pigs, given a full complement of the other necessary factors, could jeopardise the health of their litters, and it is essential to determine the significance of these findings in relation to the economic importance of haemolytic disease. This problem can be approached first by considering the clinical evidence from the field.

The losses resulting from haemolytic disease could be direct and obvious, as when new-born pigs die shortly after birth, or they could be secondary and somewhat masked, as would be the case if there were a widespread predisposition to intercurrent infection or the conventional anaemia of older suckling pigs. As yet, there is no evidence on any secondary effects and these cannot be discussed further. The evidence concerning mortality or the number of pigs that are affected clinically, suggests that the disease is uncommon. Even allowing for the fact that the disease is relatively new and few practitioners have experience of the condition, it appears to be considerably less common than the number of sows with high antibody titres.

To determine whether this impression is true, it will be necessary to examine more closely the sequence of events that are initiated by a high titre of maternal iso-antibodies and culminate in a clinically sick baby pig. Briefly, these events are: the transfer of antibodies to the colostrum and thence to the piglet's serum, the sensitization of the piglet's red cells, the consequent acute haemolysis and other pathological changes and the ability of the baby pig to counter these harmful effects. The ultimate extent and severity of the disease is determined by factors operating at each of these stages and there are, therefore, many theoretical reasons why a high incidence of antibodies could be associated with a low incidence of clinical disease.

*This figure represents the lowest titre of iso-antibodies that we have found in the serum of sows when they had litters clinically affected with haemolytic disease but, as our clinical experience of this condition is limited to less than 20 sows, this standard is rather arbitary.

In order to understand the factors that modify the above sequence of events, a great deal of knowledge is still required—of which the nature of the haemolytic process is but one example. It is unlikely, therefore, that rapid progress will be made along these lines. However, of all the investigations that would yield such basic information, there is one that conspicuously requires attention. This concerns the question of the individual red-cell antigen systems and their inheritance. At many points in the epidemiology and pathogenesis of this disease, it is indispensable to know the character of the antibody/antigen systems involved, and the absence of information on the blood groups of pigs (other than the A - O system) is probably the greatest handicap to a more fundamental approach to this problem.

In the meantime, some progress could be made on an ad hoc basis by examining litters of sows found to have a high titre of iso-antibodies and ascertaining the incidence of pigs with strongly sensitized cells and clinical symptoms. Pending further investigations on these points, some facts are unquestionable. As the vaccination programme embraces more of the existing unvaccinated herds, so the number of animals at risk must increase proportionally. Furthermore, it is virtually certain that the incidence of the condition must increase with time, for the pigs already vaccinated will receive additional injections.

Finally, the association between the use of crystal violet vaccine and red cell iso-antibodies does not preclude the existence of naturally occurring haemolytic disease of pigs by transplacental sensitization.

CONCLUSIONS

Injections of crystal violet swine fever vaccine experimentally into young pigs stimulated the production of red cell iso-antibodies. After repeated injections, the titre of these antibodies in some pigs reached a high level.

Examination of the sera from pigs in the field showed that a very large proportion of animals that had received more than one prophylactic injection of crystal violet swine fever vaccine had a high titre of red cell immune iso-antibodies.

The production of these high titred iso-antibodies in sows may render them liable to have litters affected with haemolytic disease due to blood group incompatibility.

ACKNOWLEDGMENTS

This work has been aided by a general financial grant from the Agricultural Research Council and by a joint personal grant to one of us (R.R.A.C.) from the Nuffield Foundation and the Agricultural Research Council.

Messrs. J. C. Buxton and N. H. Brooksbank supplied us with the foundation stock of clinical cases, and Mr. Buxton has continued to help us by directing our attention to cases in the field. Mr. H. I. Field has like-

wise introduced us to herds where the disease occurred clinically. The unvaccinated herds were from the practices of Mr. F. D. Stephenson and Mr. R. P. Webber, and to all these colleagues we express our thanks.

The task of collecting blood samples, particularly in the field, has been made very much easier by the efforts of Mr. P. J. D. V. Brett, who has handled all types of pigs with determination and efficiency, often in difficult circumstances.

Finally, we are indebted to Professor L. P. Pugh for initiating this work and supporting it actively with many accessory facilities.

REFERENCES

Buxton, J. C., and Brooksbank, N. H. (1953a) *Vet. Rec.*, **65**, 287; (1953b). *Nature, Lond.*, **172**, 355.

Kershaw, G. F. (1950). *Vet. Rec.*, **62**, 383.

Saison, R., Goodwin, R. F. W., and Coombs, R.R.A. (1955) *J. comp. Path.*, **65**, 71.

ADDENDUM

Since this paper went to press the sera of 39 more unvaccinated pigs have been tested for iso-antibodies against the cells of pig S7. In none of these sera were antibodies demonstrated. This brings the number of control sera to 147 while within this total the number possessing iso-antibodies (see Figs. 1 and 2) has not increased.

[*Received for publication, June 21st,* 1954]

Peace in the Kennels

Canine hysteria, epilepsy and other nervous conditions, can quickly affect the smooth running of the kennels. The risk of such undesirable disturbances, however, is reduced to the minimum by treating "suspect" boarders with 'Mysoline' during their stay.

A new and highly effective anticonvulsant, 'Mysoline' combines the advantages of powerful activity with extremely low toxicity, and freedom from hypnotic effect. Being virtually tasteless, it is very easy to administer, and is readily taken by animals.

'MYSOLINE'

Trade Mark

in the treatment of nervous disorders and complications

IMPERIAL CHEMICAL (PHARMACEUTICALS) LIMITED

A subsidiary company of Imperial Chemical Industries Ltd. WILMSLOW, MANCHESTER

Ph.462

GURR'S

Established 1915

STANDARD STAINS AND REAGENTS FOR MICROSCOPY

KNOWN AND USED

THROUGHOUT

THE WORLD

WRITE for any of the following literature :

Catalogue of stains B15

Gurr's Commentary No. 12

Q.R. Laboratory Notes

Biological Staining Methods, 5/6d. post free.

Catalogue of Accessories A.C. 1953

GEORGE T. GURR LTD.

Laboratories :

136/8 NEW KINGS ROAD LONDON, S.W.6

No connexion with any other business of similar name

The Journal

of

Comparative Pathology

and

Therapeutics

is worth advertising in, for its subscribers are influential and in all parts of the world.

	Per Issue	*Per Volume (4 issues)*
Full page	£5 0 0	17 0 0
Half page	£2 15 0	9 7 0
Quarter page	£1 10 0	5 2 0

Published quarterly by the
UNIVERSITY PRESS OF LIVERPOOL
LIVERPOOL 3. ENGLAND

THE BRITISH VETERINARY JOURNAL

(Established 1875)

A MONTHLY REVIEW OF VETERINARY SCIENCE

RECENT CONTRIBUTIONS *include the following papers*

S. N. NANDI. An Investigation into Bovine Hæmaturia in Kalimpong (District Darjeeling), India.

A. E. HARROP. Artificial Insemination of a Bitch with Preserved Semen.

BJORN SIGURDSSON. Paratuberculosis (Johnes Disease) of Sheep in Iceland.

BJORN SIGURDSSON. Rida, A Chronic Encephalitis of Sheep.

L. H. AYNSLEY and D. L. HUGHES. Neomycin as a treatment for Bovine Mastitis.

W. C. EVANS, E. T. REES EVANS and L. E. HUGHES. Studies on Bracken Poisoning in Cattle.

R. TUCKER and R. MILLAR. The Pattern of Nervous Symptoms in the *Chondrodystrophic* Dog.

W. M. MCKAY. The Feeding of Antibiotics to Farm Animals.

S. A. BUNCE. Observations on the Blood Sedimentation Rate and the Packed Cell Volume of some Domestic Farm Animals.

Annual Subscription 40*s*

Published by:

BAILLIÈRE, TINDALL & COX LTD.

8 Henrietta Street, Covent Garden, London, W.C.2